Official Organ of the Society for the Study of Development and Growth

GROWTH

A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

VOLUME XVII

PAPERS 428 - 445

1953

Science
Q1
84
A1
G8

Science Library

QP 84 A1 G88

Official Organ of the Society for the Study of Development and Growth

GROWTH

A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

U of O
MAR 26 1953
SCIENCE LIBRARY

VOLUME XVII, NUMBER 1

PAPERS 428-432

MARCH, 1953 **$6.00 A YEAR**

Entered as second class matter on October 20, 1941, at the Post Office, Worcester, Mass., under the Act of March 3, 1879.

GROWTH

A journal for the coordination of studies of increase and development as general properties of nature. Published by contributors and subscribers on a cooperative, non-profit, non-salary basis. The field of investigation is primary and work will be published in which the approach is through mathematics, physics, chemistry, genetics, embryology, philosophy, and anthropology; just as will studies of development in its several expressions, and of mass increase and the part played therein by nutrition, vitamins, and hormones.

It is published four times a year during March, June, September and December at $6.00 per volume (outside U. S. and Canada, $6.50). Single numbers $2.00. There will be one volume of at least 400 pages each year.

All manuscripts and subscriptions should be sent to: The Editors of Growth, Lankenau Hospital Research Institute, Fox Chase, Philadelphia 11, Pa.

THE EDITORS OF GROWTH

Managing Editor
STANLEY P. REIMANN, M.D.
Lankenau Hospital Research Institute and Institute for Cancer Research, Fox Chase, Philadelphia, Pa.

Editor-in-Chief
IRENE COREY DILLER, PH.D.
Institute for Cancer Research, Fox Chase, Philadelphia, Pa.

Donald P. Costello, Univ. of N. Carolina
S. A. Courtis, University of Michigan
Earl L. Green, Ohio State University
W. W. Greulich, Stanford University
F. S. Hammett, Lankenau Hospital Research Institute
Leigh Hoadley, Harvard University
Warren H. Lewis, The Wistar Institute
Leo Loeb, Washington University
Willard C. Olson, University of Michigan, Ann Arbor, Michigan
Otto Rahn, Idaho State College
Earle Reynolds, Atomic Bomb Casualty Comm'n, APO #182, San Francisco, Cal.
E. W. Sinnott, Yale University
Philip R. White, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

Corresponding Editors

B. Boysen Jensen, Univ. of Copenhagen
Ivar Broman, Tornblad Institutet
Luigi Castaldi, Università di Cagliari
A. Dalcq, Université de Bruxelles
E. Fauré-Fremiet, Collège de France
Alexander Haddow, Chester Beatty Research Institute
Julian S. Huxley, Zoölogical Society
Jacques Monod, Institut Pasteur
Joseph Needham, Caius College
Yo. K. Okada, Kyoto Imperial University
G. Teissier, Faculté des Sciences
A. H. Roffo, Universidad de Buenos Aires

Growth, 1953, **17**, 1-6.

ORGAN:BODY WEIGHT, PROTEIN:ORGAN AND FAT:ORGAN RATIOS IN LARGE ALBINO RATS[1]

FREDERICK SPERLING

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service—Federal Security Agency, Bethesda, Maryland

(Received for publication November 12, 1952)

During a study of the excretion and distribution of C^{14}-labeled carbonate and formate (12), it was found necessary to collect data dealing with the ratios of organ weights to body weights, of proteins to organ weights, and of fats to organ weights. A review of the literature revealed few such data in general, and none for large rats. It is thought that it might be helpful to others to present the data thus gathered in the course of the experiments, although the conditions reported are not usual for such determinations.

MATERIALS AND METHODS

The data refer to 11 male Osborne-Mendel rats, 12 to 18 months old, weighing between 400 and 537 grams, with a mean weight of 475 grams. Five rats weighed more than 500 grams.

Each animal was kept for eight days in a sealed glass metabolism cage of 12-liter capacity. Since all excreta, including respiratory CO_2 was continuously collected, it was necessary to exclude atmospheric CO_2. Thus the cage could not be opened to provide food. Accordingly, an exclusively liquid diet was introduced through a tube closed with a stopcock. The diet consisted of 10% dextrose by weight in whole milk, and was similar to that used by Armstrong (2) in his experiments with radioactive carbonates. The rats were fed on this diet for 10 days to two weeks prior to each experiment. No attempts were made to measure the food intake.

At the end of the experiment the animals were weighed and sacrificed by an intraperitoneal injection of 4 mg./100 gm. of body weight of pentothal sodium. Before death, 7 to 10 ml. of heart blood was

[1]I would like to express my appreciation to Robert McLaughlin and especially to Walter S. Cool, for technical assistance.

withdrawn. The internal organs were removed, slit open, blotted, and weighed immediately. The gastrointestinal tract was divided into three parts: the stomach and esophagus, the small intestine, and the large intestine and cecum. All visible adhering fat was removed, and the tract slit, washed, blotted, and weighed. The central nervous system was removed as a unit and divided into two parts. The brain portion included the cerebrum, cerebellum, pons, and medulla. The spinal cord was taken as that portion from below the medulla to the cauda equina. Both lungs, both kidneys, and both testes, were used. In each case all visible adhering fat was removed, except that in some lungs the lymphoid tissue adhered so tenaciously that some small portions remained on the lung. The pelt, without the tail skin, was cleaned of underlying fat and muscle tissue. Conditions did not permit weight determinations of the remainder of the animal.

Immediately after weighing, the tissues were divided into smaller sections and dehydrated in 95% alcohol. These were then cut into pieces, about 1 x 3 mm. in size, and the fat extracted in a Wiley tube with absolute alcohol (3). The alcohol-fat solution was evaporated to dryness and the residue reextracted with petroleum ether, which was evaporated, and the fat dried to constant weight. For the determination of protein, the fat-free tissue was ground and treated first with 40%, then with 10% trichloracetic acid, washed and dried to constant weight. This method was used to permit comparison with Armstrong's results.

The fat and proteins of muscle were obtained from combined aliquot portions of the vastus and abdominal musculature. A square from the dorsal portion of the pelt was removed for analysis. A portion of the perirenal fat was used for the analysis of depot fat.

Results

Table 1 shows the organ:body weight ratios. The internal organs and the pelt comprise 20% of the body weight. The results of other investigators indicate that the remainder of the animal totals about 80% of the body weight, of which muscle represents 45% (5, 9), skeleton, 11% (5), depot fat, 12% (10, 11) and blood, 5% (8). More than 3% of the total body weight is represented by the combined weights of the other viscera, such as the pancreas, prostate, epididymes, bladder, eyeballs, and lenses (5). Recorded weights for the skin of the tail, which was not included in the pelt weight listed

TABLE 1
ORGAN:BODY RATIOS
(Per cent)

Organ	Mean	Range	Coefficient of[1] Variation (%)
Liver	3.09	3.5 -2.7	7.8
Heart	0.32	0.38-0.26	12.5
Lungs	0.53	0.81-0.36	26.4
Spleen	0.27	0.38-0.21	18.5
Kidneys	0.58	0.70-0.47	12.1
Stom. + Esoph.	0.46	0.57-0.37	13.0
Sm. Intestine	1.14	1.62-0.62	26.3
Lg. Int. + Cecum	0.81	1.13-0.48	24.7
Testes	0.77	0.85-0.65	7.8
Brain	0.42	0.48-0.35	9.5
Spinal Cord	0.19	0.24-0.16	10.5
Pelt[2]	11.6	13.0 -9.3	10.1
Total	20.18		

[1]$\frac{\text{Standard Deviation}}{\text{Mean}}$

[2]Ten animals used.

Note: All others based upon 11 animals (Mean wt. 475 gm.).

in Table 1, could not be found, nor could recorded weights for claws, foot pads, lymph nodes, and pinna be found. It is not unreasonable to assume that these would represent a sufficiently large percentage of the body weight to bring the total to 100%.

Webster, Liljegren, and Zimmer (13) reported that the livers of 400-gram rats weighed 4.04% of the body weight when etherized and 3.66% when bled. The kidneys weighed 0.74% and 0.72%, respectively. The ratios for liver and kidneys in Table 1 are lower than these, but they represent considerably larger animals. Phillips and Gilder (9) found that the liver in rats weighing 332 grams represented 3.4% of the body weight. Donaldson's ratios for these organs, for rats weighing 435 grams, are higher than any of the figures cited. He reported the liver to weigh 4.17% of the body weight and the kidneys to be 0.79%. The spleen ratios given in Table 1 agree with the 0.26 per cent of Webster *et al.* and of Donaldson.

Latimer (7) points out that lungs with various lesions have higher weight ratios than those which are entirely clear. The lungs of few rats are without lesions, and since none of the lungs of the rats used in these experiments were entirely clear, the considerable variation in the weight ratio was not unexpected. However, the ratios agree with those of Donaldson's 0.54%.

The washing and subsequent blotting described above, in the preparation of the gastrointestinal tract, were such that variations greater than in other tissues would be expected. The figures given in Table 1, for the entire tract, are considerably below the 3.96% given by Donaldson, who simply expressed the intestinal contents and did not wash the tract.

Freudenberger's (6) reported ratio of 2.06% is lower than Donaldson's and that of Table 1, although he used Donaldson's technique.

Freudenberger's ratios for the integument, 15% to 16%, appear to include the skin of the tail. With this consideration, the ratio for the pelt given in Table 1 is in close agreement. Donaldson, however, gave a figure of 18% for the integument.

TABLE 2
PROTEIN:ORGAN WEIGHT AND FAT:ORGAN WEIGHT RATIOS

A Protein:Organ Weight Ratios (Per cent)				B Fat:Organ Weight Ratios (Per cent)		
Coef. of Var(%)[1]	Range	Mean	Organ	Mean	Range	Coef. of Var(%)[1]
16.2	19.3 -11.0	16.2	Liver	4.5	6.7- 2.3	29.6
7.6	24.1 -18.5	22.1	Muscle	2.8	4.0- 2.0	23.3
12.7	20.5 -13.7	16.9	Heart	3.7	8.0- 2.3	48.6
21.9	18.9 - 9.0	14.3	Lungs	4.8	7.6- 0.84	44.8
14.2	19.9 -12.3	16.9	Spleen	2.6	3.2- 0.93	28.1
21.4	23.0 -11.5	17.0	Kidneys	3.6	5.1- 2.1	26.4
24.1	21.6 -10.8	14.2	Stom. + Esoph.	3.9	5.0- 2.1	23.5
22.6	13.3 - 7.3	9.4	Sm. Intestine[2]	4.5	7.1- 2.1	39.3
23.0	11.8 - 5.4	8.8	Lg. Int. + Cec.	3.4	6.9- 2.1	53.2
14.3	10.6 - 6.2	8.4	Testes[3]	2.3	2.7- 2.2	7.0
9.1	12.5 - 9.5	10.4	Brain	9.1	10.7- 7.5	11.1
8.0	8.3 - 6.5	7.5	Spinal Cord[4]	17.4	20.3-14.7	10.7
16.8	30.3 -18.6	24.7	Pelt[5]	5.5	6.5- 4.6	15.4
32.3	1.68- 0.82	1.24	Depot Fat	67.9	76.9-61.3	10.3

[1]Standard Deviation / Mean
[2]Eight animals used.
[3]Seven animals used.
[4]Nine animals used.
[5]Five animals used.
Note: All others based upon 10 animals.

Table 2A shows the percentage of proteins in the various organs listed in Table 1. The values for heart and kidneys agree closely with the 16.9% and 17.6% of Addis *et al.* (1) who used 250-gram male rats, but the liver values are lower than the 19.9% reported by these

authors. Luck (8) found the values for liver were 16.6%, for kidneys were 15.8%, and for muscle were 20.7%, for 250-gram male and female rats on a low-protein diet. These ratios are similar to those in Table 2, but Luck's value for the concentration of protein for the intestine, 13.9%, was considerably higher.

It can be seen, in Table 2A, that the greatest variation in protein between animals were found in the gastrointestinal tract, the kidneys, the lungs, and in the proteins isolated from the depot fat. In almost every case the range was greatly increased by disparate weights in one or two animals, with the remainder closely grouped. Muscle and pelt had the greatest ratios of proteins, while the gastrointestinal tract, the central nervous system, and the testes had the lowest.

Table 2B shows that the percentage of fat of the testes, brain, spinal cord, pelt, and depot fat varied relatively little among the animals, while the ratios of the fats in the other organs varied considerably. The spinal cord had almost twice the amount found in the brain, which in turn had far more than that found in any other organ. It is realized that the ether-extractable materials from the central nervous system differ considerably from the fat of other tissues. The ratios among the other organs were quite similar.

Summary

1. Despite the rather extreme experimental conditions, comparative figures of organ:body weight ratios presented here and those reported by others for smaller animals do not show great disagreement. Where comparisons are not noted, the results within the group of animals vary within reasonable limits.

2. The pelt and skeletal muscle had the highest protein ratios followed by kidney, heart, spleen, liver, lungs and stomach. The brain, the large and small intestine, the testes, spinal cord and depot fat had the lowest protein ratios.

3. The spinal cord and brain had the highest fat ratios. The ratios among the other tissues were similar to each other and were half or less the ratio of the brain fat.

References

1. Addis, T., Poo, L. J., Lew, W., & Yuen, D. W. 1936. Gravimetric methods for the determination of total body protein and organ protein. *J. Biol. Chem.*, **113**, 497-604.

2. ARMSTRONG, W. D., SCHUBERT, JACK, & LINDENBAUM, A. 1948. Distribution of radioactive carbon administered as carbonate in the body and excreta of mature rats. *Proc. Soc. Exp. Biol. Med.*, **68**, 233-240.

3. BERLIN, N. I., HUFF, R. L., VAN DYKE, D. C., & HENNESSY, T. G. 1949. The blood volume of the adult rat as determined by iron^{59} and phosphorus^{32} labeled red cells. *Proc. Soc. Exp. Biol. Med.*, **71**, 176-178.

4. BLOOR, W. R. 1943. Biochemistry of Fatty Acids and Their Compounds, the Lipids. Reinhold Publishing Corp., New York, pp. 387.

5. DONALDSON, H. H. 1924. The Rat. Data and Reference Tables. Mem. Wistar Inst. Anat. and Biol., Second Ed. Rev., Philadelphia, pp. 469.

6. FREUDENBERGER, C. B. 1932. A comparison of the Wistar albino and the Long-Evans hybrid strain of Norway rat. *Am. J. Anat.*, **50**, 293-349.

7. LATIMER, H. B. 1943. The prenatal growth of the cat. XIII. The weights of the lungs, trachea and larynx in the fetal and in the adult cat. *Growth*, **7**, 239-250.

8. LUCK, J. M. 1936. The question of protein storage. *J. Biol. Chem.*, **115**, 491-509.

9. PHILLIPS, R. A., & GILDER, H. 1940. Metabolism studies in the albino rat. The effect of hypophysectomy on the percentage of body weight as muscle. *Endocrinology*, **27**, 597-600.

10. REED, L. L., YAMAGUCHI, F., ANDERSON, W. E., & MENDEL, L. B. 1930. Factors influencing the distribution and character of adipose tissue. *J. Biol. Chem.*, **87**, 147-174.

11. ANDERSON, W. E., & MENDEL, L. B. 1932. Factors influencing the distribution and character of adipose tissue in the fat. I. The effect of ovariectomy and of feed-thyroxine. *J. Biol. Chem.*, **96**, 307-312.

12. SPERLING, F., MAXWELL, E. S., & VON OETTINGEN, W. F. The comparative excretion and distribution of C^{14}-labeled carbonate and formate in large albino rats (in preparation).

13. WEBSTER, S. H., LILJEGREN, E. J., & ZIMMER, D. J. 1947. Organ-body weight ratios for liver, kidneys and spleen of laboratory animals. I. Albino rat. *Am. J. Anat.*, **81**, 477-513.

Growth, 1953, **17**, 7-20.

EFFECT OF 2,000 ROENTGENS LOCAL X-IRRADIATION ON THE GROWTH OF RAT BONE

S. H. COHN AND J. K. GONG

Clinical Radiobiology Branch, Biological and Medical Sciences Division, U. S. Naval Radiological Defense Laboratory, San Francisco 24, California

(Received for publication November 24, 1952)

INTRODUCTION

While the relative radiosensitivity of bone during growth has been demonstrated by numerous investigators (1, 2, 3, 4, 5), only a few attempts at quantifying these effects of ionizing radiation on bone growth have been carried out (6, 7, 8). It is the object of the present investigation to study both the qualitative and the quantitative effects of radiation on bone growth in the rat.

It has been found in a previous experiment that whole body x-irradiation of young rats with 500 roentgens produced only temporary bone damage (9), although the animals exhibited acute radiation illness and loss of body weight. The bone damage was reflected in a decrease in the rate of growth of tibia (both in weight and length), in the irradiated animals.

The marked changes in bone resulting from partial body x-ray treatment, which are described in the literature, occur after much larger doses than the relatively small dose of total body irradiation that results in death (500-600 roentgens). In order to increase the applied dose to the bone without producing generalized harmful effects involving the remainder of the animal, it was decided to administer a dose of 2,000 roentgens x-irradiation to one hind leg.

EXPERIMENTAL

The experiment was designed so that one tibia of each of a large group of young rats of uniform age was treated with 2,000 roentgens x-irradiation. These animals were then followed up to adult life (160 days), and the growth of both tibiae and the tibiae of a similar group of control animals was then studied, measurements being made at regular intervals under standardized conditions.

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

An experiment (Experiment 1) was performed with 120 animals, 60 of which received x-irradiation over the left tibia, and 60 of which were kept as non-irradiated controls. The same type of experiment (Experiment 2) was repeated at a later date with 54 animals, all of which were irradiated as the above mentioned 60 animals. The animals used were male Sprague-Dawley rats. Those in the first experiment were approximately 41 days of age at the time of irradiation, while the animals of the second experiment were approximately 39 days of age.

Six animals were placed in each experimental group, and six animals in each control group, for each of the ten sacrifices, in Experiment 1. Six animals were placed in each experimental group for each of the nine sacrifices in Experiment 2.

The animals were anesthetized and placed in a lead cylinder, with their left hind leg protruding through a hole. Each of the experimental animals was exposed to 2,000 roentgens over the left hind leg. The field of irradiation covered the distal end of the femur, all of the tibia-fibula, and the entire foot. The remainder of the animal was protected with ¼ inch of lead shielding.

Dosage factors used were: 250 KVP, 15 ma, 35-cm. target-to-skin distance, 9.3 minutes exposure, 0.5 mm. Cu and 1 mm. Al.

After irradiation the animals were placed two in a cage, and fed *ad libitum* throughout the experiment. Food and water were withheld for the twenty hours preceding sacrifice.

Both right and left tibiae were removed immediately after sacrifice. They were dissected clean, weighed, and measured in the longest dimension in length. The bones were put in a drying oven at 100°C overnight. The dry residue was weighed (dry weight), and after being placed in a muffle oven set at 500°C overnight, again weighed (ash weight). The difference between the dry weight and the ash weight is taken as the weight of the matrix, representing the dehydrated organic portion of the bone.

Results

The local effects of irradiation were observed by comparing the length, weight, matrix weight and bone ash percentage of the irradiated tibiae with those of the non-irradiated homologous tibiae. Systemic effects were observed by comparing the above measurements in the non-irradiated homologous tibiae of the irradiated animals (homol-

ogous control tibiae), with the tibia of the normal non-irradiated animals (control tibiae).

Gross Effects

The irradiated rats ate well, and suffered no observable illness as a consequence of the irradiation. At the conclusion of the experiment, the animals seemed entirely normal, except for shortening of the irradiated limb.

Gross examination of the limbs of the irradiated animals revealed no local soft tissue damage resulting from the application of 2,000 roentgens to the left hind leg, although, in every case, the irradiated hind limb exhibited epilation from the fifteenth day through the completion of the experiment on the 120th day post-irradiation.

The body weights of the non-irradiated control animals and the animals which received the local body irradiation both follow the same normal growth curve (Figure 1*). The control tibiae and the homologous control tibiae exhibit no significant difference in any of the measurements of bone growth used throughout the period of the experiment.

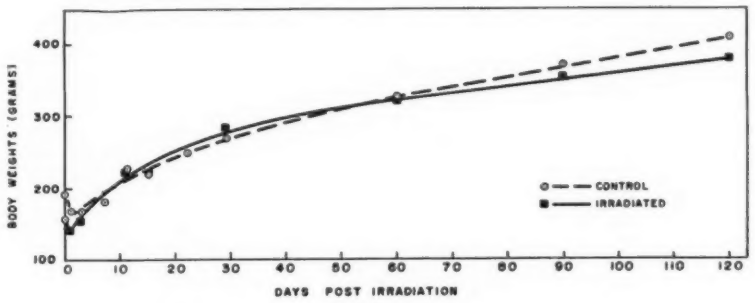


FIGURE 1
EFFECT OF 2,000 ROENTGENS PARTIAL BODY X-IRRADIATION ON THE BODY WEIGHT

Quantitative Effects

The lengths and weights of the organic and inorganic components of the normal and the irradiated tibiae of Experiments 1 and 2 are summarized in Tables 1 and 2.

*In Figure 1, and the figures to follow, only the mean values for each day of the experiment (average of six rats), are shown for clarity.

TABLE 1

EFFECTS OF 2,000 ROENTGENS X-IRRADIATION TO THE TIBIA, EXPERIMENT I

Summary of Data

Day	Final Body Wt. gm.		Tibia														
			Length mm.			Dry Weight mg.			Ash Weight mg.			Matrix mg.			Bone Ash Percentage		
	C	X	C	XR	XL	C	XR	XL	C	XR	XL	C	XR	XL	C	XR	XL
1	169	143	35.0	32.5	33.0	190	169	175	104	95	95	86	74	81	54.7	56.2	54.0
3	169	154	34.7	33.7	34.1	201	205	195	115	104	109	86	101	86	57.2	50.7	55.9
7	176	197	34.5	36.6	35.2	219	238	244	119	136	131	100	102	100	54.3	57.0	56.2
11	226	224	36.9	36.9	35.8	270	290	264	154	166	151	116	124	113	56.8	57.2	57.2
15	220	221	36.6	37.0	34.3	266	288	235	153	164	138	108	107	97	57.2	62.8	51.5
22	249	249	37.7	39.3	36.5	309	311	274	185	180	165	124	131	109	59.8	58.0	60.2
29	267	282	38.6	38.4	36.5	338	343	306	201	219	191	137	158	115	59.4	54.0	57.8
60	326	323	41.3	41.0	31.8	420	425	314	277	270	209	143	155	105	65.8	63.5	66.6
90	371	353	43.2	43.0	34.1	509	498	373	317	309	237	192	189	136	62.3	62.0	63.5
120	408	379	43.7	43.4	32.8	552	511	359	350	327	228	202	184	131	63.5	64.0	63.4

C = Control animal, left tibia.
XR = Experimental animal, right tibia, non-irradiated.
XL = Experimental animal, left tibia, irradiated with 2,000 r.
Each value represents the mean for six rats.

TABLE 2

EFFECTS OF 2,000 ROENTGENS X-IRRADIATION TO THE TIBIA, EXPERIMENT II

Summary of Data

Day	Final Body Wt. gm.	Tibia									
		Length mm.		Dry Weight mg.		Ash Weight mg.		Matrix mg.		Bone Ash Percentage	
	X	XR	XL	XR	XL	XR	XL	XR	XL	XR	XL
1	116	30.5	30.3	145	150	76	80	69.1	69.3	52.5	53.7
3	123	31.2	31.2	164	169	88	92	75.9	77.6	53.5	54.1
7	152	33.0	32.0	187	162	100	102	87.6	86.8	53.3	54.2
15	193	34.5	32.2	214	186	127	114	86.8	71.1	59.4	61.7
22	218	36.8	32.6	284	236	166	144	118	92.0	58.5	61.0
29	249	38.1	33.3	338	278	198	164	140	114	59.0	59.0
60	313	40.3	32.6	441	353	280	224	161	128	63.5	63.5
90	345	42.2	35.4	495	374	299	223	196	151	60.3	59.7
120	366	42.9	34.1	522	382	339	253	183	129	65.0	65.8

C = Control animal, left tibia.
XR = Experimental animal, right tibia, non-irradiated.
XL = Experimental animal, irradiated with 2,000 r.
Each value represents the mean for six rats.

TABLE 3
RELATIONSHIPS BETWEEN THE VARIABLES OF BONE GROWTH

(1) *Length of bone as function of body weight*

$\log L = \log a + b \log W$
L = length of bone, mm.
W = body weight, gm.

	Control	XR_1	XR_2	Average
a	8.32	8.09	7.90	8.10
b	0.28	0.28	0.28	0.28

$S_y = \pm 1.0$ mm.
$r = 0.97$

(2) *Ash weight of bone as function of body weight*

$\log A = \log a + b \log W$
A = ash weight of bone, mg.
W = body weight, gm.

	Control	XR_1	XR_2	Average
a	0.09	0.16	0.13	0.13
b	1.38	1.28	1.33	1.33

$S_y = \pm 13.9$ mg.
$r = 0.99$

(3) *Matrix weight of bone as a function of body weight*

$\log M = \log a + b \log W$
M = matrix weight, mg.
W = body weight, gm.

	Control	XR_1	XR_2	Average
a	0.87	0.88	0.69	0.81
b	0.90	0.91	0.95	0.92

$S_y = \pm 9.3$ mg.
$r = 0.97$

(4) *Ash weight of bone as a function of matrix weight*

$\log A = \log a + b \log M$
A = ash weight, mg.
M = matrix weight, mg.

	Control	XR_1	XR_2	Average
a	0.09	0.08	0.12	0.10
b	1.58	1.60	1.53	1.57

$S_y = 30.3$ mg.
$r = 0.93$

Control = non-irradiated controls, Experiment I
XR_1 = homologous controls, Experiment I.
XR_2 = homologous controls, Experiment II.
S_y = standard error.
r = Brevais-Pearson coefficient of correlation.

In an analysis of growth, it was found by Huxley (10) and by Needham (11) that growing components can be expressed as power functions in terms of body weight. These results were confirmed experimentally by Zucker (12) in a study on rat bone. The transformation from a plot of the growth variables against age to a plot of the log of these variables against the log of body weight provides a linear relationship which can be readily expressed and analyzed statistically. Further, in normal rats, the growth of the bones is correlated more closely with the growth of the body as a whole than with the age of the animal, so that by making the above-mentioned transformation the effect of the normal variations among animals of the same age is reduced to a minimum (12).

The data, for the control animals, therefore, have been expressed as a series of straight line functions whose slopes and intercepts are presented in summary form in Table 3.

It was found that the variation in the data obtained on the irradiated limbs was considerably greater than that for the controls. Moreover, the irradiation produced a disturbance to the normal growth functions, so that no single law appears to hold over the period of time studied. Because of these differences, analysis of the growth of the irradiated components is not entirely analogous to that of the control components. Whenever possible, however, similar analysis was performed so that direct comparison could be made.

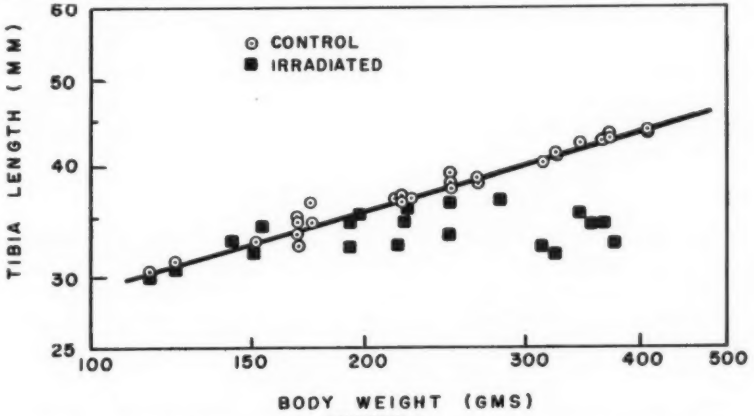


FIGURE 2

EFFECT OF 2,000 ROENTGENS PARTIAL BODY X-IRRADIATION ON TIBIA LENGTH

A. *Length of tibia.* The growth curves for the tibia length of the normal and the irradiated animals are shown in Figure 2. It can be seen that the log of the length of tibiae of the control animals and the homologous control animals both exhibit a linear relationship with the log of the body weight, as expressed by the equation:

$$(1) \qquad \log L = \log 8.10 + 0.28 \log W$$

where L is length of the bone in mm., and W is body weight in gm.

There is no significant difference in the above data between the controls and the homologous controls (Table 3). The standard error of the above curve, based on the values of all the controls, is 1.0 mm., and the coefficient of correlation is 0.97.

This relationship between length and body weight is disturbed following irradiation. From the tables or from plot of length against time, it can be seen that the irradiated tibiae do not differ significantly from the controls during the first three days post-irradiation. By the end of the first week, however, the rate of growth in length is depressed, and by the 15th day post-irradiation, the irradiated tibiae are significantly shorter in length than the controls. After 30 days post-irradiation the slope of the growth curve is decreased essentially to zero. At the conclusion of the experiment the irradiated tibiae are about 25% shorter than the normal control tibiae.

The general contour of the bones in which growth was inhibited appears unchanged from the homologous controls.

B. *Weight.* A plot of the log of the inorganic or ash weight of the bones against the log of the body weight results in a straight line function, indicating that for the control and homologous control tibiae a power function is obtained (Figure 3). This function is expressed by the equation:

$$(2) \qquad \log A = \log 0.13 + 1.33 \log W$$

where A is the ash weight in mg. and W is the body weight in gm.

The ash weights of the irradiated tibiae do not appear to follow a power function after the first eleven days. From the 15th day the slope of the curve for the irradiated tibiae drops off considerably. From 90 to 120 days the rate of growth of the ash weight drops to zero.

The organic portion of the bone, or matrix, behaves very similarly to the inorganic portion of the bone. For the control and homologus

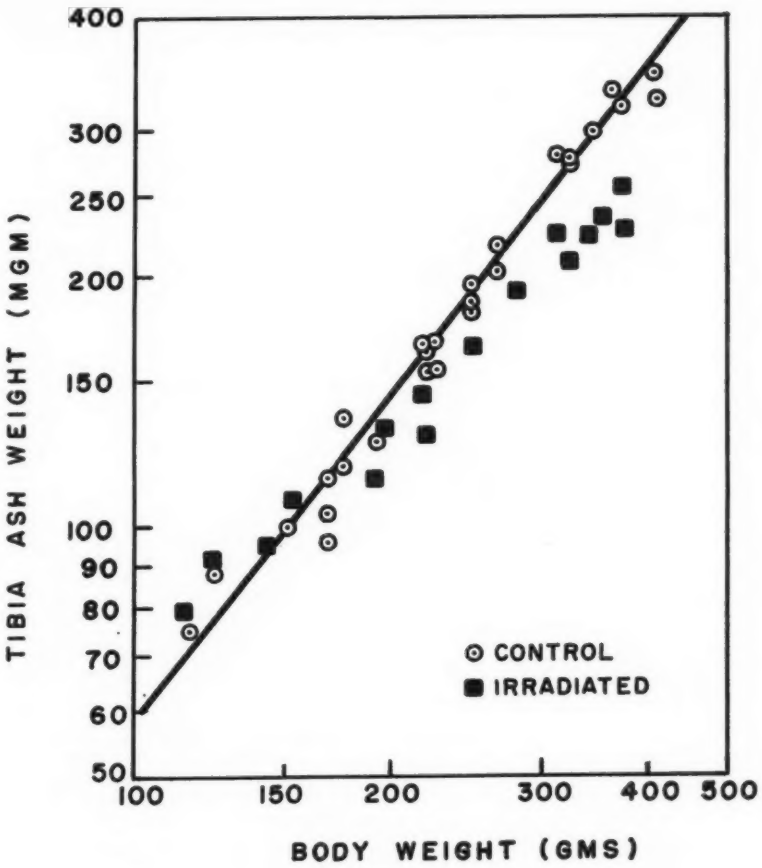


FIGURE 3
EFFECT OF 2,000 ROENTGENS PARTIAL BODY X-IRRADIATION ON THE TIBIA ASH WEIGHT

control tibiae a linear function is obtained when log of matrix weight is plotted against log of body weight (Figure 4). This can be expressed by the equation:

$$\log M = \log 0.81 + 0.92 \log W \quad (3)$$

where M is the matrix weight in mg. and W is the body weight in gm.

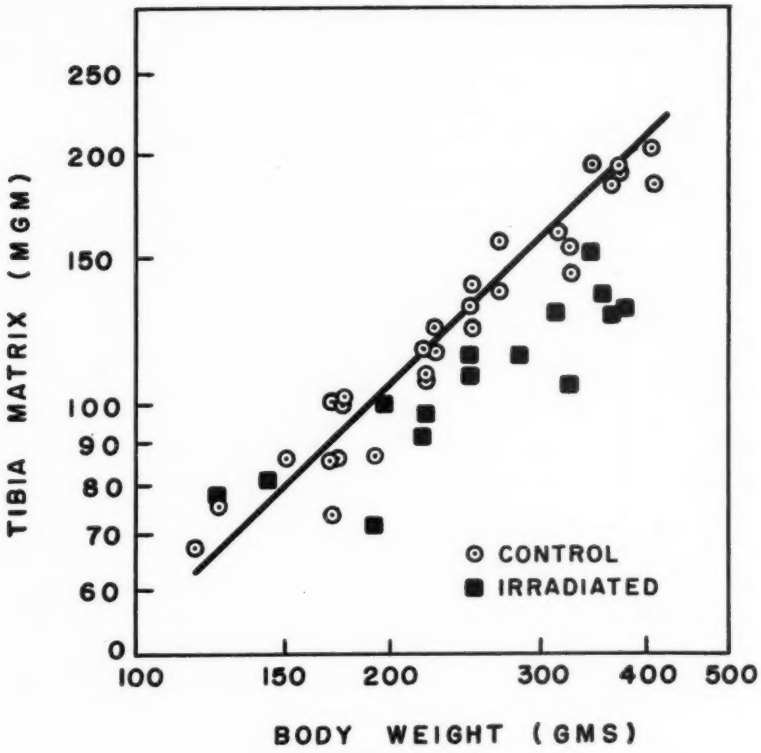


FIGURE 4

EFFECT OF 2,000 ROENTGENS PARTIAL BODY X-IRRADIATION ON THE TIBIA MATRIX WEIGHT

The weights of the irradiated tibia matrices cannot be characterized by a single power function.

The relationship between ash weight and matrix can best be seen by a graph relating the two quantities, as shown in Figure 5. It can be seen that the relation of log of ash weight to log of matrix is a power function. The controls and the homologous controls for the matrix weights show no significant variation (Table 3).

In the irradiated tibiae, the ratio of the log of ash weight to log matrix is the same as that of the controls for the first three weeks.

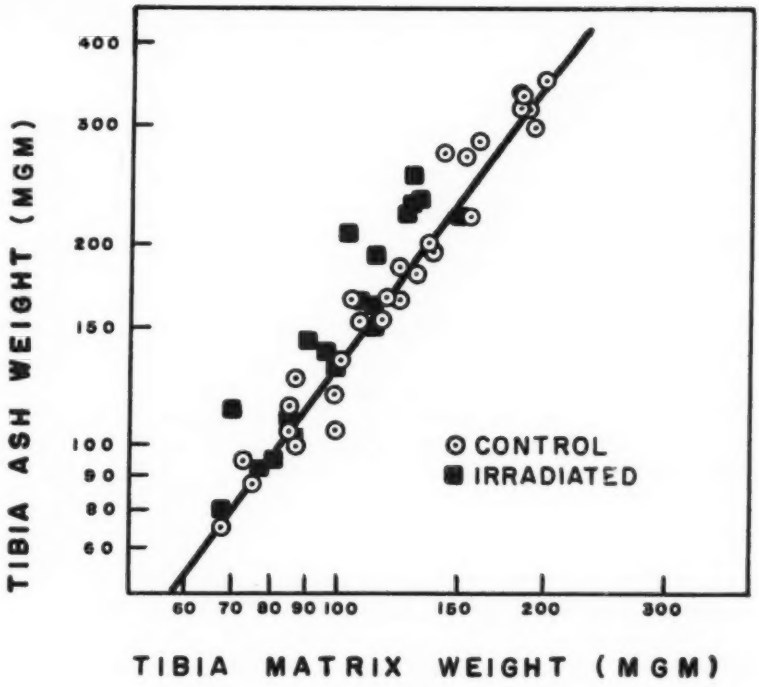


FIGURE 5

EFFECT OF 2,000 ROENTGENS PARTIAL BODY X-IRRADIATION ON THE RELATIONSHIP BETWEEN TIBIA ASH AND MATRIX WEIGHTS

Following this, however, the matrix weight drops off much more rapidly than the ash weight, so that the value of ash weight for a given value of matrix is slightly higher in the irradiated tibiae than in the control tibiae at all times after the first three weeks.

C. *Bone Ash Percentage.* The bone ash percentage (ash weight/dry weight) of the tibiae is shown in Figure 6. It can be seen that this ratio increases from 53% to approximately 65% in the control animals during the 120-day period of the experiment. The irradiated tibiae show no significant differences from the controls.

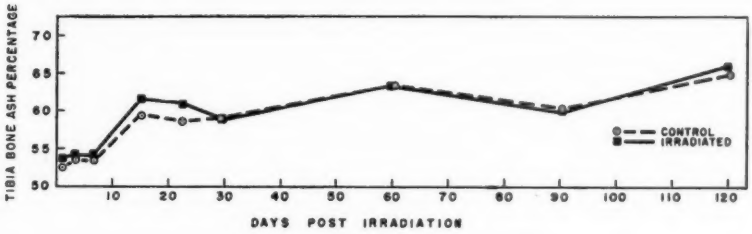


FIGURE 6
EFFECT OF 2,000 ROENTGENS LOCAL BODY X-IRRADIATION ON THE TIBIA BONE ASH PERCENTAGE

DISCUSSION

Following a radiation dose of 2,000 roentgens to one limb, only local effects on the animal were observed. These effects were confined to the skin and to the bone in the irradiated area.

Since no significant differences appeared between any of the measurements of growth of the homologous control tibiae and those of the tibiae of the non-irradiated control animals it must be assumed that there is no systemic effect acting on the homologous control tibiae following irradiation of one limb.

Quantitative Effects of Irradiation on Bone Growth

While the quantitative appraisal of irradiation effects in growing animals is difficult, the study of the growth of the bones of young growing animals of a uniform age and weight group provides an excellent medium for this investigation. Rapid changes in the weight of mineral constituents and organic matrix normally take place during this period of the animal's life, and deviations in the rate of growth yield quantitatively reproducible data on the alteration of these biological processes under the influence of radiation.

The growth processes as measured by criteria such as length, ash weight and matrix weight appear to be quantitatively similar in normal and irradiated tibiae for the initial post-irradiation period. Later the irradiated tibia growth is impaired and finally there is a cessation of growth. Thus the period of local bone growth is shortened by exposure of the bone to irradiation. The data would indicate that resorption of bone is occurring at a rate equal to or greater than that of bone formation. This is analogous to the situation found in older animals reaching the end of their growth period, that is, the bone resorption process predominates.

The growth in weight of the organic portion of the bone, or matrix, responds to irradiation quantitatively the same as do the inorganic salts of bone. The log of ash weight and log of matrix plotted against the log of body weight provide very similar curves, differing only in the slope. In normal rats the inorganic salts are deposited in bone at a greater rate than that at which the matrix is formed (see slopes in Table 3). In the irradiated tibiae the matrix weight falls off more rapidly than the ash weight following irradiation which may be due to a greater radiosensitivity or to a faster turnover time of the matrix.

Contrary to findings reported in early references (3, 13), no decalcification of bone or condensing osteitis was observed following a large dose of x-irradiation. At no time did the bone ash percentage of the irradiated tibiae differ from the non-irradiated tibiae. The decrease in bone growth following irradiation entails the simultaneous disappearance of organic and inorganic components of bone. As pointed out under the section "RESULTS", the bone ash percentage increases slightly but constantly in the young growing animal. This would indicate that as the animals mature, the bones become better calcified.

As the depression of growth continues long after the short period of irradiation, the effect of irradiation on growth can be analyzed in terms of destruction of growth capacity. Since the results indicate that the growth capacity for the tibiae lie (at least in part) within the bone itself, it must be determined by one or more of the components of bone. These components are most likely cellular and the diminution in growth capacity thus would result from the destruction of these cells by irradiation.

Summary

The present investigation presents the quantitative effects of x-irradiation on bone growth.

One leg of each of a group of young rats was x-irradiated with 2,000 r. These animals were sacrificed at intervals throughout their active growth period. The local effects of the irradiation were observed by comparing the inorganic weight, organic weight, length of bone and bone ash percentage of the irradiated tibiae with the corresponding measurements in the non-irradiated homologous control tibiae. Possible systemic effects to the non-irradiated homologous tibiae of the irradiated animals were sought by comparing their meas-

urements with the growth measurements of the tibiae of the normal non-irradiated animals.

It was found that a dose of 2,000 roentgens administered to one limb produced chiefly local effects in the skin and the skeletal tissue of the area irradiated. There were no significant differences in any of the above measurements between the homologous control tibiae and the tibiae of the non-irradiated animals.

Bone ash percentage is not altered after 2,000 roentgens local irradiation, indicating that local x-irradiation does not produce decalcification as such though there is a proportionate resorption of "whole" bone including matrix and inorganic salts.

Bone growth in the normal rat can be expressed according to well defined laws, the equations for which are presented. No single law appears to hold for the irradiated bones over the experimental period. The growth of bone per unit of body weight was found to be disturbed by irradiation. This retardation in osteogenesis following irradiation appears to be permanent and irreversible at the level of dosage employed; no renewal of growth activity occurred in the four-month period of the study.

References

1. Perthes, G. 1903. Ueber den einfluss der rontgenstrahlen auf epithelial gewebe inzbesondere auf das carcinom. *Verhandl. d. deutsch. gesellsch. f. chir.*, **32**, 525-532.
2. Forsterling, K. 1906. Ueber allgemeine und partielle wachsthumssterungen nach kurz dauernden rontgenbestrahlugen von saugethieren. *Arch. f. klin, chir.*, **81**, 505-515.
3. Latarjet, H. 1912. Resultats experimentaux sur l'accroissement des os en longeur. *Compt. rend. de anat.*, **14**, 72-80.
4. Dahl, B. 1934. Effets des rayons x sur les os longe en developpement; etude radiobiologique et anatomique chez le rat. *J. de Radiol. et d'Electrol.*, **18**. 131-140.
5. Bisgard, J. D., & Hunt, H. B. 1936. Influence of roentgen rays and radium on epiphyseal growth of long bones. *Rad.*, **26**, 56-64.
6. Regen, E. M., & Wilkins, W. E. 1936. The effect of large doses of X-rays on the growth of young bone. *J. Bone & Joint Surg.*, **18**, 61-68.
7. Brooks, B., & Hillstrom, H. T. 1933. The effect of roentgen rays on bone growth and bone regeneration. *Am. J. Surg.*, **20**, 599-614.
8. Hinkel, C. L. 1942. The effect of roentgen rays upon the growing long bones of albino rats. *Am. J. Roentgenol. & Radium Therapy*, **47**, 439-457.
9. Cohn, S. H., & Gong, J. K. Effect of 500 roentgens whole body x-irradiation on the growth and metabolism of rat bone. (In press.)
10. Huxley, J. S. Problems of Relative Growth. (Methuen, 1932). 276 p.
11. Needham, J. 1939. Chemical heterogeny and the ground-plan of animal growth. *Biol. Rev.*, **9**, 70-109.
12. Zucker, T. F., & Zucker, L. M. 1946. Bone growth in the rat as related to age and body weight. *Am. J. Physiol.*, **146**, 585-592.
13. Ewing, J. 1926. Radiation osteitis. *Acta Radiol.*, **6**, 399-413.

Growth, 1953, **17**, 21-44.

THE EFFECTS OF THIOUREA ON LIMB REGENERATION IN THE TADPOLE[1]

ANNIE M. PEADON

School of Nursing Education, The Catholic University of America, Washington, D. C.

(Received for publication January 5, 1953)

INTRODUCTION

Although young tadpoles regenerate lost hind limbs quite readily, adult frogs have no such ability. The fact that the loss of regenerative capacity occurs as a feature of metamorphosis was first noted by Spallanzani (1769). The exact metamorphic stage at which the regenerative ability is lost differs in different species of anura (Liosner, 1931; Borssuk, 1935; Polezaiev, 1936; Forsyth, 1946) and there is also variation in regenerative capacity depending upon the level of amputation of the limb (Marcucci, 1916; Schotté and Harland, 1943).

A number of investigations have been conducted to ascertain the factors involved in this loss of regenerative capacity and as a result several hypotheses have been advanced to explain the phenomenon on the basis of factors of either local or systemic origin.

The local changes in the limb tissues which may influence the regenerative ability at late metamorphic stages are of several types. Schotté and Harland (1943) have suggested that the connective tissue which they observed to form over the cut end of the bone in non-regenerating limbs might act as a mechanical block to the regenerative process. Rose (1944, 1945) and Gidge and Rose (1944) presented evidence that dedifferentiation in amputated limbs over which adult skin is grafted is limited to muscle and marrow, but that limb stumps covered with larval skin show dedifferentiation of all tissues. Explanations based on local changes resulting from greater de-

[1]A contribution from the Department of Biology, The Catholic University of America, Washington, D. C. This paper is based on the author's dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The writer wishes to express her appreciation to Professor W. Gardner Lynn who suggested this problem to her and under whose guidance it was done. Thanks are also expressed to Rev. Henry E. Wachowski and Dr. John A. O'Brien, who served on the dissertation committee.

grees of differentiation of the limb tissues at late stages have also been supported by observations of Guyenot (1927), Naville (1927), Polezaiev (1936, 1946) and Goodwin (1946).

Forsyth (1946) has presented histological evidence that the immediate cause of regenerative failure is the formation of an abnormal blastema or of no blastema at all.

Evidence that there is some general systemic change during metamorphosis which affects regenerative ability has been given by Borssuk (1935), Polezaiev and Ginsberg (1939) and Polezaiev (1946). It was found that transplantation of a limb from an animal which still showed complete regenerative ability to one in which this capacity had been lost resulted in loss of regenerative ability in the transplanted limb. This was ascribed to differences in the humoral medium at the two different stages. Because the loss of the ability to regenerate limbs occurs during or shortly after metamorphosis, some workers have been led to seek the explanation in a direct action of the increased thyroxin level which occurs at this time and which is known to be responsible for the metamorphic process itself. Thus, Pawlowsky (1923) found that in salamanders which are fed thyroid, the rate and degree of limb regeneration were decreased. Speidel (1929) reported inhibition of regeneration of limbs in tadpoles by thyroid administration, if treatment was instituted before or at the time of amputation. If, however, the treatment was deferred for seven days, regeneration was accelerated. These findings have been confirmed by Warren and Bower (1939) and by Siegel (1951).

The findings of these workers, using animals in the hyperthyroid condition, thus indicate that the increased level of thyroxin in some way inhibits the regenerative process, providing the increased level is attained before the onset of regeneration. The reverse type of experiment, production of a hypothyroid condition, to study the effects on limb regeneration has not been done. Such a study has been made for tail regeneration (Ghidoni, 1948) and indicates that the regeneration of the tadpole tail is influenced by thyroid hormone. The problem of loss of regenerative capacity at late metamorphosis cannot be studied in such material, however, since at late metamorphic stages the tadpole tail is undergoing autolysis as a feature of the metamorphic pattern. In the present investigation *Rana pipiens* tadpoles of various ages have been treated with thiourea and the effects of the resulting thyroid inhibition upon limb regeneration have been

studied. In view of Forsyth's findings, special attention has been given to the histological aspects of the regenerative process.

Materials and Methods

Tadpoles of *R. pipiens* were obtained by the method of pituitary-induced ovulation. The larvae were kept in tap water in white enameled pans measuring 9 x 12 x 2 inches. When the animals became active feeders, large individuals of uniform size were selected and divided into groups of 10 animals per pan. The culture medium was changed and the animals were fed daily. The diet consisted of strained spinach, vegetable and liver soup, and sphagnum moss. All animals were kept under identical conditions of light, temperature, and food supply. When the desired growth stages were reached amputations of limbs were made near the mid-shank of the left hind limb, using MS-222 in concentration of 1:3,000 as an anesthetic.

One group of experiments was set up using animals which were in early metamorphosis, while the second group involved animals in late metamorphosis. The stages selected for a particular series of experiments were based on the degree of external differentiation of the limbs and corresponded to those used by Forsyth (1946) for *R. sylvatica*.

Thiourea was administered by placing the larvae in a 0.05% solution of the drug in tap water. Animals used for histological studies were fixed in Bouin's fluid. After fixation of the entire animal the limbs were removed, washed in water, dehydrated in cellosolve, cleared in benzene, sectioned at 10 micra and stained with Gallagher's hematoxylin followed by Mallory's triple stain.

Observations

I. Effects of thiourea treatment upon the course of limb regeneration in early stages of metamorphosis.

A. *Preliminary Studies*

Forsyth's (1946) studies have demonstrated that tadpoles of *R. sylvatica* in which the hind limb shows very early differentiation exhibit complete regeneration of limbs amputated at the level of the mid femur. In this stage, which he designates stage 1b, the limb has as yet no visible knee joint and the foot is represented by a pad which may or may not have toe rudiments.

The first series of experiments in the present study dealt with animals in this stage. One set of 20 animals, which constituted the control set, was kept in tap water throughout the experiment. The other set (20 animals) was placed in 0.05% thiourea solution immediately after amputation of the limb, and maintained in this solution continuously. Both sets were operated upon on the same day. The animals were examined daily for 10 days and every 2-3 days thereafter. Both the amputated and the unamputated limbs of each animal were measured and observations on the degree of differentiation were recorded.

The averages of the measurements of the unoperated limbs of the control and experimental series are given in Table 1, and it will be

TABLE 1

EFFECTS OF 0.05% THIOUREA ON LIMB GROWTH IN UNOPERATED LIMBS (AVERAGE MEASUREMENT IN MM.)

Post-operative Day	6	8	9	11	14
Controls	3	3.6	3.7	4.0	5.4
Treated with 0.05% thiourea	3	3.5	3.5	3.5	3.6

noted that after the 8th day there was little increase in the length of the limbs of the animals that received treatment. During this period individual measurements within a group never varied more than 1.5 mm. for the experimentals nor more than 3 mm. for the controls. After 32 days the average length of unoperated limbs of the experimental group was 4.4 mm., while in the control group the average length was 24.4 mm. Six of the experimental animals had been lost by this time. It is clear that a 0.05% thiourea solution is effective in inhibiting the thyroid function in these animals and that, at this particular stage, the inhibition of hind limb growth by this treatment first becomes apparent about 8 or 9 days after the beginning of treatment.

The cut surfaces of the amputated limbs exhibited rapid healing, which was usually accomplished by the end of the first day. This was followed on the second day by the formation of a small projecting tip in the center of the stump. This tip appears to be over the end of the cartilaginous shaft. By the 6th day the tip has led to the formation of a cone. This cone was evident in all except two animals, one of which was in the experimental group and one in the control group.

In the regenerating limbs of the controls, the events after cone formation closely resemble the pattern seen in normal limb development. A pad, much as is seen in the normal limb of Forsyth's stage 1b, is formed, and this is followed by indications of a single toe rudiment. The formation of the toe rudiment may or may not be preceded by the appearance of visible blood vessels forming a circuit in the area of the future rudiment. Shortly after the appearance of the first rudiment, others differentiate and webbing gradually appears. The limb meanwhile undergoes a steady increase in length.

All regenerating limbs in this series showed cones on the 8th day and pad formation was evident on the 11th day. There was no discernible difference in either rate or degree of regeneration in the experimental animals as compared with the controls up to the time of pad formation. The subsequent differentiation of the limbs, however, was markedly inhibited in the treated group. By the 17th day, 19 of the 20 control animals showed three or more toe rudiments, whereas only five of the experimental animals had any toe rudiments and these showed only one or two rudiments. As long as thiourea was administered, the experimental group showed little differentiation beyond one or two rudiments. The toes were well differentiated by the 24th day in the control group. After 36 days the thiourea was discontinued in the experimental group. Only five animals in this group survived beyond the 40th day, but these showed gradual resumption of differentiation.

These experiments would seem, at first sight, to indicate that thyroid inhibition has no effect on the early stages of regeneration, but inhibits later differentiation in both degree and rate. However, if it is remembered that eight days were required before evidence of inhibition of growth in the unoperated limbs was noted and that by this time all operated limbs showed cone formation, the possibility suggests itself that the thyroid was not inhibited in these experiments until after the regenerative process was well under way. It seems clear that later differentiation was greatly decreased in both rate and degree by inhibition of the thyroid, but this is true for the unoperated limbs as well as the regenerating ones. It simply indicates a cessation of the metamorphic process.

In view of these facts it was decided to test the effect of instituting thiourea treatment 10 days prior to amputation of the limb to assure inhibition of the thyroid at the onset of the regenerative process.

Animals so treated shall hereafter be referred to as "pretreated" animals. Only three animals were used for this experiment so that detailed individual observations could be carried out. The three animals were carefully selected in stage 1b and were placed in separate containers. One of these was placed in 0.05% thiourea, the other two being kept in tap water. At the end of 10 days amputations were performed on all three animals. The "pretreated" animal was then returned to tap water while one of the previously untreated animals was now placed in 0.05% thiourea. The series thus consisted of:

Animal 1. Control (untreated).

Animal 2. Treated with 0.05% thiourea, treatment beginning at the time of amputation (hereafter referred to as the "treated" animal).

Animal 3. Treated with 0.05% thiourea for 10 days prior to amputation. Treatment discontinued at time of amputation ("pretreated" animal).

Although these animals were at Forsyth's stage 1b when first selected, by the time the operations were performed they had reached stage 2b which Forsyth describes as follows: "The knee joint is apparent, the shank forms an obtuse angle with the thigh and the toe rudiments are well differentiated." This stage corresponds to stages XII and XIII of Rugh (1951).

The animals were examined and camera lucida drawings of the limbs were made daily for 19 days. At the time of amputation, the limbs of the "pretreated" animal were slightly less well developed than were those of the other two animals; the knee joint, however, was apparent. After discontinuance of treatment, the unoperated limb gradually increased in length and degree of differentiation. Limb growth became more pronounced the 5th day after the administration of the drug was discontinued.

The first and second days after operation the stumps were marked by healing in all animals. It was not possible to distinguish any differences in the course of regeneration of the limbs of the three animals during this period.

On the 4th day after amputation all animals showed a tip projecting from the end of the stump, and all appeared about the same. On the 5th day, however, the tip remained on only the control. The limb of the pretreated animal had taken on a more pad-like appearance. By the 7th day the operated limbs of all animals showed pad formation.

The first indication of digit formation was seen on the 9th day. At this time digit circulation was seen in the pretreated animal. Neither of the other animals showed any evidence of digit formation. The rudiment was quite clearly seen by the 10th day, and on the 11th day all animals showed the formation of one digit.

Consideration of this series in conjunction with the first series brings out the following points: The first series indicated that 8 to 10 days are required for 0.05% thiourea treatment to produce inhibition of growth in unoperated limbs. It may, therefore, be presumed that the failure of treated animals to show any effects on the early stages of limb regeneration was due to the fact that their thyroids were not effectively inhibited during this period. Late stages of regeneration were markedly affected, the thyroid inhibition now being produced. The "pretreated" animal of the second series shows precisely the reverse of this condition. As judged by growth of the unoperated limb, this animal had effective thyroid inhibition at the time of operation and for about 6 days thereafter. Following this it appears that the thyroid level was probably restored to normal. Since this "pretreated" animal showed an acceleration of the early phases of regeneration such that digit formation occurred on the 9th day as compared with the 11th day for "treated" and control animals, we may tentatively conclude that thyroid inhibition during the early phases of regeneration permits a speeding up of the process.

B. *Histological Studies*

In the light of the foregoing experiments it was felt that a histological study of the course of regeneration under the various conditions of treatment outlined above was now indicated. Animals were selected so as to be in Forsyth's stage 2b at the time of amputation.

The following experimental series were set up:

1. Thirty control animals.
2. Thirty animals placed in 0.05% thiourea solution at the time of amputation. ("Treated" animals.)
3. Thirty animals placed in 0.05% thiourea solution 10 days prior to amputation. Treatment discontinued at the time of amputation. ("Pretreated" animals.)

One animal from each series was removed each day for 20 days. The specimens were examined for external evidence of limb regeneration, camera lucida drawings of the limbs were made and the animals were preserved for histological study.

Histological examination of the shank at the time of operation (stage 2b) reveals that the tibia-fibula is not fused, the cartilaginous skeleton is well laid down with a distinct perichondrium and periosteal bone collar. The mid-diaphysis shows pre-ossification changes; myoblasts are arranged in distinct bundles and muscle cell orientation is evident. A few myofibrillae are demonstrable in the shank myoblasts.

The histological findings of Forsyth (1946) indicate that the loss of regenerative capacity in limbs is accompanied by either the formation of an abnormal blastema or the formation of no blastema at all. If a blastema does form, a mitotic rate lower than normal is found during the proliferative phase. In view of these facts, and the findings reported in the previous section, special emphasis has been placed on these two aspects in the present study.

Because of the small size of the limbs at the time of operation it was not possible to make the amputation at the same level in each instance. This was apparent in the later histological examination. This sometimes made evaluation of the results difficult in view of the fact that Schotté and Harland (1943) have clearly demonstrated that the regenerative ability of tadpole limbs varies with the level of amputation. However, the level of amputation was always considered when making comparisons.

Forsyth (1946), describing the histology of regenerating limbs of *R. sylvatica,* pointed out that the stump is first covered by epidermal cells one or two layers thick and then a "piling up" of cells occurs. It was found in the present study that epidermal healing is complete in 24 hours and by the end of two days the epidermal cells vary from 9-10 layers thick in some areas to 14-15 layers in other areas. The degree of "piling up" apparently has no relationship to thyroid inhibition, as variation occurred in all animals.

In addition to these epidermal changes, many blood cells are seen in the amputation area in all stumps examined at the end of the first day. There is little change on the second day except that all stumps show changes in the cartilage matrix in the region of the amputation level. It appears to be pulling away slightly from the distal perichondrium and also degenerating at the cut surface.

Figures 1, 2 and 3 show the histological features that are present on the third day. The control (Figure 1) and the treated animal (Figure 2) show no blastema at this time. Careful study of the

sections under high power reveals that much of the cartilage matrix has been lost, but that the cells maintain their original orientation. It will be noted (Figure 3) that the limb of the pretreated animal shows a distinct blastema. Close observation of this blastema reveals fragments of what appears to be cartilage matrix. However, the cells are not confined to the area previously occupied by cartilage, but rather are somewhat diffuse. The origin of these cells was not determined, but from the appearance of the cartilaginous shaft during early blastema formation it seems certain that they are in great part derived from the cartilage.

It should be pointed out that the level of amputation in this particular "pretreated" animal was near the distal end of the tibia-fibula, whereas the limbs of the control and the treated animal were amputated at the proximal end of the calcaneum and astragalus. When it is remembered that normally the regeneration rate is decreased with higher level amputations, the presence of a blastema would seem to be even more significant. It was not only precocious in appearance, but present at a higher limb level.

On the 5th day the control animal shows a blastema. The level of amputation on this animal was the proximal end of the calcaneum and astragalus. The treated animal, in which the amputation level was the mid tibia-fibula, shows no blastema. This situation is difficult to interpret, but it is possible that the presence of the blastema in the control is due to the more distal level of amputation. The blastema in the "pretreated" animal at this time is rather extensive. By the end of the 6th day all animals show blastemata. At this time the blastema is much more extensive in the "treated" and "pretreated" animals than it is in the control. The level of amputation and the extensiveness of the blastema in the control animal compares rather well with what is seen in the "pretreated" animal on the third post-operative day.

Rose (1948) has described in some detail the migration of epidermal cells into the blastema area in regenerating limbs of *Triturus viridescens*. In the present material, at about the time of the appearance of a blastema or shortly thereafter, the integrity of the inner border of the epidermis appears to be lost, and there is strong indication that epidermal cells are contributed to the blastema. Many mitotic figures are seen in the epidermis in this area and the loss of cells and the interruption of the border is always seen in an area

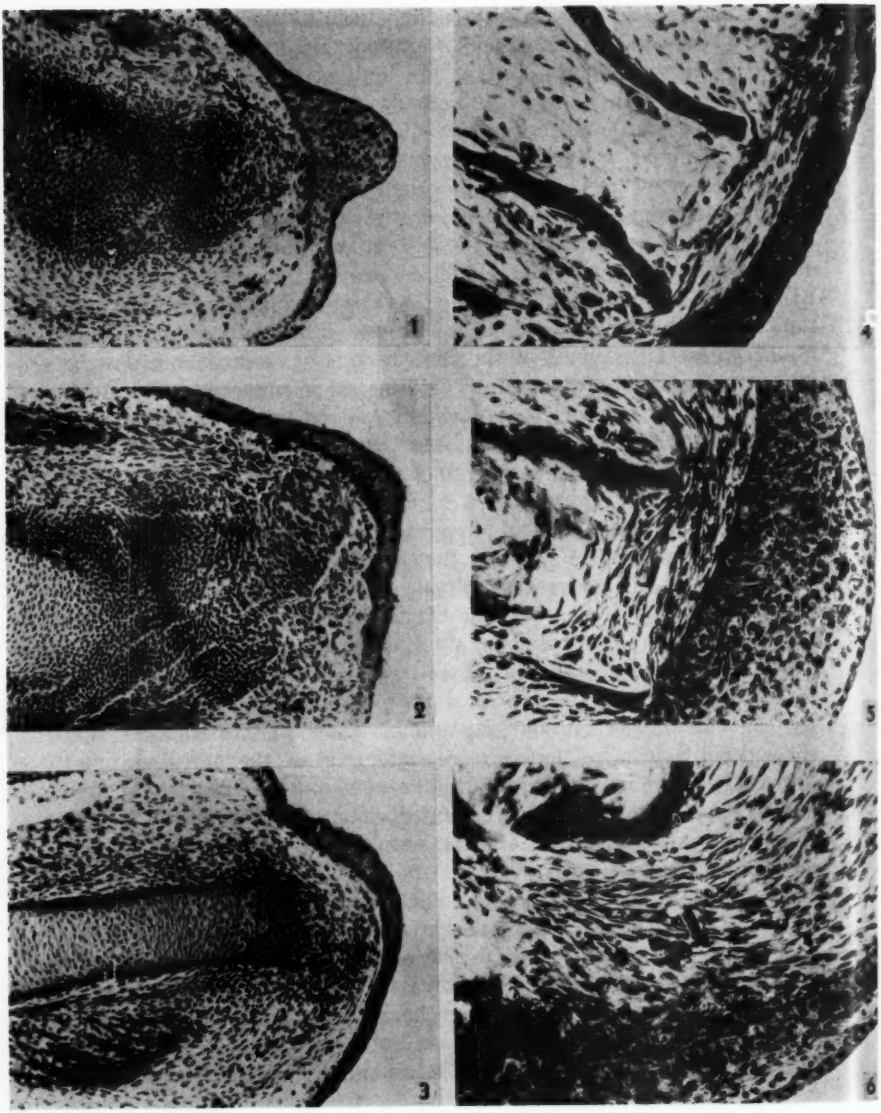

1
4
2
5
3
6

PLATE I

All figures are unretouched photomicrographs

All sections figured are of ***Rana pipiens,*** cut at 10 micra, and stained with Gallagher's hematoxylin followed by Mallory's triple stain.

1. Longitudinal section through limb of control animal in early metamorphosis 3 days after amputation. (stage 2b). X150.
2. Longitudinal section through limb of animal in early metamorphosis 3 days after amputation. Treatment with 0.05% thiourea was begun at the time of amputation. (stage 2b). X150.
3. Longitudinal section through limb of animal in early metamorphosis 3 days after amputation. Treatment with 0.05% thiourea for 10 days prior to amputation. (stage 2b). X150.
4. Longitudinal section through limb of control animal in late metamorphosis 6 days after amputation. (stage 3b). X300.
5. Longitudinal section through limb of animal in late metamorphosis 6 days after amputation. Treatment with 0.05% thiourea was begun at the time of amputation. (stage 3b). X300.
6. Longitudinal section through limb of animal in late metamorphosis 6 days after amputation. Treatment with 0.05% thiourea for 10 days prior to amputation. (stage 3b). X300.

which shows some degree of "piling up" and the absence of a dermis. This phenomenon does not seem to be related to the thyroxin level as it is seen in the controls as well as the experimental animals. However, it seems slightly less prominent in the "treated" animals.

The histology of regenerating limbs during the period in which regenerative capacity is lost, has been described by Forsyth (1946). He found that limbs in this stage, which he calls the "critical" period, form an abnormal blastema and furthermore that the mitotic index in such a blastema is decreased. It, therefore, appeared that a study of the mitotic index in animals with thyroid inhibition should prove valuable.

Mitotic indices were determined for all blastemata through the 10th day. At this time differentiation was under way. In the counts all cells clearly in prophase were included as well as those in later stages of mitosis. The mitotic index is based on counts of 1,000 cells for each blastema, the index being an expression of the number of cells in mitosis per 100 cells counted.

Table 2 shows the mitotic indices in relation to post-operative day. It will be observed that the "pretreated" animal shows a relatively low index until the 6th post-operative day. This fact is probably highly significant when one recalls that the animals gave evidence of recovery from the thiourea treatment 5-6 days after treatment was discontinued. It would seem, then, that a decreased level of thyroxin is accompanied by decreased mitotic activity in the normal blastema. When the thyroxin level is restored the mitotic index is increased. This would seem to be in conformity with the findings of Speidel, that thyroid extract administered after the onset of proliferation increases the rate of differentiation and growth of regenerating limbs.

Table 3 shows the mitotic indices in relation to the blastema age. This gives a better comparison of the mitotic activity in the three groups of animals. It will be noted that the control animal and the "treated" animal compare rather well in mitotic activity. The initial lag in activity in the "pretreated" animal shows up clearly in this table. The failure of the "treated" animals to show a decreased rate is probably explained by the requirement of 8-10 days for thiourea to produce sufficient inhibition to cause decreased growth rate in the limbs. Actually the limbs are showing differentiation in the form of accumulation in areas of future cartilage by the time the thiourea is effective.

Thus it seems clear that blastema formation is hastened in animals in which the thyroxin level has been decreased by the administration of thiourea. The mitotic activity is decreased by inhibition of the thyroid and is increased as the gland recovers, indicating that mitotic activity is regulated at least in part, by the thyroxin level. Because

TABLE 2
MITOTIC INDICES OF BLASTEMATA ACCORDING TO POST-OPERATIVE DAY

Post-operative Day	3	4	5	6	7	8	9	10
Control			5.7	5.0	3.2	2.4	6.1	4.3
"Treated"				6.1	5.2	3.5	4.2	5.4
"Pretreated"	2.7	3.0	3.3	6.0	7.1	6.3	6.1	3.2

TABLE 3
MITOTIC INDICES OF BLASTEMATA ACCORDING TO AGE OF BLASTEMA

Blastema Day	1	2	3	4	5	6	7	8
Control	5.7	5.0	3.2	2.4	6.1	4.3		
"Treated"	6.1	5.2	3.5	4.2	5.4			
"Pretreated"	2.7	3.0	3.3	6.0	7.1	6.3	6.1	3.2

the cells of the early blastema seem to be formed in great part from dedifferentiated cartilage, it would seem that the precocious appearance of the blastema may possibly be associated with an increased rate of dedifferentiation of cartilage.

II. Effects of Thiourea Treatment on Limb Regeneration in Late Metamorphic Stages

Consideration will now be given to the effects of thiourea treatment in animals which have reached metamorphic stages at which the capacity for limb regeneration is normally lost.

Forsyth found that tadpoles of *R. sylvatica* with limbs in stage 3a or later had lost the capacity to regenerate limbs amputated at the mid-thigh level. In the present material it was found in preliminary studies that *R. pipiens* larvae with limbs in stage 3b do not normally regenerate limbs when the amputation level is the mid-shank.[2] The animals chosen for the present series of experiments were at this stage at the time of amputation. The limbs show an acute angle between the thigh and shank, with a well developed shank and foot. The

[2]In preliminary studies, Forsyth found that larvae of *R. pipiens* lost the power to regenerate limbs that were amputated in the mid-thigh during stage 1b of limb development. In the present material, where the amputations were made at the more distal mid-shank level, regeneration occurred at stage 3a but not at stage 3b.

forelimbs can be felt beneath the operculum at this stage. Stage 3b of Forsyth corresponds to stages XVI, XVII, or XVIII of Rugh.

The series consisted of the following experiments:

1. Control animals (47 animals).
2. Animals placed in 0.05% thiourea at the time of amputation (8 animals) or one day (19 animals) or two days (34 animals) before amputation.
3. Animals treated with 0.05% thiourea for 10 days prior to amputation (44 animals).

It is recognized that because of the fact that the animals in this series are in a later stage of metamorphosis, at which time there is a higher thyroxin level in the blood, treatment with thiourea may not bring about the same degree of inhibition of limb growth as was seen in the younger animals. It seemed likely that a longer period of treatment would have resulted in a greater variation in the metamorphic stages obtained. Moreover, since the studies reported above were made on the limb tissues in which the decreased thyroxin had been operating for 10 days, it was decided to treat these animals for the same length of time.

Two animals were removed from each group daily for 10 days, every other day through the 18th day and at longer intervals thereafter. They were examined for external evidence of regeneration, camera lucida drawings were made, and the animals were preserved for histological study.

A. *Gross Appearances*

There was no external evidence of regeneration in any of the limbs of this group during the course of the experiment (24-25 days).

B. *Histological Studies*

The normal histological appearance of the shank at the time of operation in this series was as follows. The skin is well differentiated with a well developed dermis, which equals or exceeds the epidermis in thickness. There are clearly differentiated muscle fibers with very distinct cross striations and these fibers are arranged in definite bundles. Calcification, extensive cartilage destruction, and ossification are occurring in the mid-diaphysis of the tibia-fibula.

Following limb amputation, epidermal healing at the end of one day is seen in all animals examined and is essentially as described for the

younger larvae. The thickness of the epidermis varies from 2-3 cell layers in some animals to 5-8 cell layers in others. As in the earlier stages, there seems to be no relation between this variation and thyroid inhibition. At this time the muscle fibers immediately proximal to the level of amputation take a lighter stain than those in other areas, and in some instances, nuclei are apparently being lost from the cytoplasm.

By the second day these changes in the muscle fibers are more pronounced, but there is little other change. The distal portions of the fibers seem to have lost their individuality and the cytoplasm appears to be "running together." The tip of the cartilaginous shaft is still closely covered by epithelium in all animals on the third day, with very few cells intervening. However, there are blood cells, connective tissue fibers, and what appear to be fibrocytes present within the shaft tip. The limbs of all animals present essentially the same picture, except that more blood cells are found and slight erosion of the cartilage is seen in the "pretreated" animals.

Little change is seen on the 4th day. However, a few cells and connective tissue fibers have appeared between the shaft tip and the epithelium. The dermis is absent in this region. At this time a few large stellate cells which are lighter staining and broader than the other cells in the region are seen in all stumps. Their origin cannot be determined. In some sections they appear to arise from dedifferentiating muscle fibers and in others they appear to be contributed by the periosteum. These cells are more numerous in the "pretreated" animal.

For clarity the findings after the 4th day will be presented separately for the three groups of animals in this series.

1. *Histology of Limbs of Control Animals*

By the fifth day a thin pad of cells is found between the skin and the cartilaginous shaft. The dermis is still absent here, and the cells extend into the shaft which is marked distally by an erosion of the matrix. The pad consists of long slender cells which have the general morphology of fibrocytes. Blood cells, stellate cells and loosely arranged fibers are also present. The cells seem to bear no special relationship to each other. The muscle fibers at this time appear to be dedifferentiating extensively and contributing spindle-shaped cells to the stump.

PLATE II

All sections are unretouched photomicrographs

7. Longitudinal section through limb of control animal in late metamorphosis 18 days after amputation. (stage 3b). X54.

8. Longitudinal section through limb of animal in late metamorphosis 18 days after amputation. Treatment with 0.05% thiourea was begun at the time of amputation. (stage 3b). X54.

9. Longitudinal section through limb of animal in late metamorphosis 18 days after amputation. Treatment with 0.05% thiourea for 10 days prior to amputation. (stage 3b). X54.

The pad is increased in thickness by the sixth day, and the cells and fibers appear to be aligning themselves parallel to the surface (Figure 4). The fibrocytes and stellate cells are scattered throughout the pad which contains a relatively large number of connective tissue fibers.

By the end of the ninth day, the pad of cells and fibers has distinctly the appearance of connective tissue. There are, however, a few of the stellate cells still present. In the region adjacent to the epidermis the fibers are interlacing, whereas, in the area adjacent to the shaft tip the fibers are arranged parallel to each other, and appear to enclose the shaft tip, so as to give a two-layered effect. This pad in no way resembles the blastemata seen in the younger larvae reported in the preceding section. A cartilaginous cap, which corresponds to that described by Schotté and Harland (1943) and Gidge and Rose (1944), is seen to be forming around the shaft tip at this time. The formation of this cap seems to be comparable to the formation of a callus in bone repair. The first evidence of its appearance is a proliferation of the cells of the osteogenic layer of the periosteum on the lateral aspect of the shaft. This overgrowth spreads down the shaft and eventually surrounds the tip. This differentiation of cartilage differs greatly from that seen in the younger larvae. There is never an accumulation of cells, but rather a direct transformation with rapid formation of a matrix. The cells never round up as seen in the typical precartilage cells in limbs of younger larvae.

The twelfth day is marked by an extensive cartilaginous cap. At one point between the cap and the epidermis the cells appear somewhat embryonic in that they are more rounded and have nuclei that are relatively larger and more round than the nuclei of the other cells. In one area there is a mass of these embryonic cells less definitely oriented than other cells of the stump, but many interlacing fibers are present. A few mitotic figures are seen in these cells, which seem to be contributing to the cartilaginous cap. After the twelfth day there is little change in the histology of the stumps except that the cap becomes more extensive, and the "enclosing" of the tip has disappeared by the fourteenth day. The cartilaginous cap is very extensive by the eighteenth day. Figure 7 represents the tip of the stump at this time. This particular section is such that a solid mass of cartilage is seen. Except for connective tissue and a few myoblasts, the tissue found between the cartilage and the skin is composed of cells all of

which seem to be differentiating into cartilage. These are seen as a darker staining group of cells surrounding the distal end of the shaft.

Thus, although there is regeneration of cartilage and connective tissue and possibly a few muscle cells, there is no organization into limb structures. There does seem to be some attempt at blastema formation if the stellate cells can be considered to be dedifferentiated cells. As has been pointed out, their origin was not determined, but it seems likely that they either represent dedifferentiated muscle or have migrated from the periosteum.

2. *Histology of Limbs of the Treated Animals*

The histology of the limbs of the treated animals is essentially the same as that seen in the control group, except that the pad of cells and fibers between the shaft tip and the epidermis is slightly thicker than in the controls.

3. *Histology of Limbs of the Pretreated Animals*

The pretreated animals show a slight difference from the control and the treated animal on the fifth day. Careful observation reveals that cells and fibers adjacent to the shaft tip are arranged in a definite pattern, with alignment parallel to the surface, somewhat enclosing the tip. These cells are long and slender and appear to be fibrocytes. Distal to this layer there is seen a layer of stellate cells and fibers more loosely arranged and in no definite orientation. Between these cells and the dermis there is a third layer of cells which appear smaller and more rounded than the other cells and which seem to be released from the dermis. Blood cells are also seen. Just lateral to the shaft in the same limb, this three-layered effect seems to be more prominent. The dermis is absent here. It will be recalled that in the controls the fibrocytes and stellate cells are scattered throughout the pad with no separation into layers.

These differences are more pronounced on the sixth day. A comparison of Figures 4, 5, and 6 will show the greater thickness of the pad in the pretreated animals and also the layered effect.

On the ninth day a blastema-like pad is seen. Immediately adjacent to the tip of the shaft there are a few fibers and cells with the "enclosing" orientation previously described. Just distal to this layer there is a mass of cells about 10 cells thick which have a somewhat blastema-like appearance. It should be pointed out that this area

resembles a blastema only in that the cells are more embryonic in appearance than the other cells of the stump, but they do not present the diffuse arrangement seen in the young normal blastemata. The cells always maintain a rather definite orientation and continuity with other tissues of the stump. They stain much as do the stellate cells referred to above, but seem slightly more rounded. Mitotic figures are seen in this region. It will be recalled that a somewhat layered effect was seen in the controls at this time. However, this was primarily a result of the arrangement of the fibers rather than a distinction of cell types.

On the twelfth day the blastema-like cells are still present and are more prominent than the embryonic cells described in the controls. There are fewer fibers present in this area and the cells are more diffuse than those seen in the controls. The "enclosing" effect of the cells at the tip of the shaft is absent.

There is little change by the eighteenth day and at this time these limbs appear quite similar to those of the control group (Figure 9).

Although no regeneration occurred in this group there does appear to be an alteration of the events following limb amputation. Thus, the stellate cells are present earlier and in greater number in the pretreated group. The layered effect is more pronounced, and appears in the pretreated animals on the fifth day. It did not appear until the ninth day in the control and treated animals. Furthermore, the cells as well as the arrangement of the fibers appeared in layers. The "enclosing" of the shaft tip appeared in the pretreated group the fifth day and was not demonstrable on the twelfth day or thereafter. Enclosing was not seen in the control and treated animals until the ninth day and was absent on and after the fourteenth day.

Discussion

The present study has shown that inhibition of the secretory activity of the thyroid gland in tadpoles in stages at which complete limb regeneration is normally possible causes more rapid blastema formation and probably also results in an increased rate of cartilage dedifferentiation. Mitotic activity and the rate of tissue differentiation are reduced, however. These findings are in accord with the results of some of the earlier work on the relation between the thyroid and regeneration in amphibians. Walter (1911) reported a retardation of differentiation of regenerating limbs in Triton as a result of

thyroidectomy and Schotté (1926) and Hall and Schotté (1951) found a similar retardation or even complete inhibition of regeneration after hypophysectomy in Triturus. Speidel's (1929) detailed studies of the effects of administration of thyroid extract to tadpoles during limb regeneration also gave results which are in agreement with the present findings, for they showed that an increased thyroxin level during the early stages of regeneration inhibits the regenerative process, while a high thyroxin level at later stages causes acceleration of differentiation. In the present study the thyroxin level was decreased at these same stages and precisely the reverse effects were obtained.

An attempt to determine the reason for the loss of regenerative capacity in the limbs of anura during late metamorphosis has been made in this study by experiments in which the effects of thyroid inhibition at non-regenerating stages have been investigated. It might be supposed that, since thyroid inhibition results in more rapid dedifferentiation and blastema formation in earlier stages, such treatment should also be effective at later stages, where the capacity for dedifferentiation and normal blastema formation seems to have been lost. As has been pointed out by several earlier authors, this idea would also seem indicated by the fact that the loss of regenerative capacity occurs during late metamorphosis when the thyroxin level is known to be high. As has been seen, however, the present experiments have failed to support the concept that the thyroxin level alone determines the degree of regenerative capacity in the limb. Though there was some evidence to indicate that inhibition of the thyroid stimulated an attempt to form a blastema at late stages, no true blastema ever formed. Dedifferentiation of muscle cells appeared to be unaffected by the treatment though the decreased thyroxin level did seem to favor dedifferentiation of the dermis. It is possible that a greater degree of inhibition of the thyroid might stimulate this attempt at blastema formation to a greater degree, but it seems clear that complete regeneration could not be induced by this treatment.

It appears that a high thyroxin level is accompanied by a decrease in the rate of the early phases of regeneration, but that a critical level is necessary for the subsequent phases (proliferation and differentiation) to occur. In later metamorphic stages, the tissues are unable to form a normal blastema even though the thyroxin level is decreased. This would seem to indicate that the tissues have undergone some

change during morphogenesis which adversely affects their blastema forming ability.

Gidge and Rose (1944) have given evidence that loss of regenerative capacity is associated with a loss of dedifferentiation ability. They maintain that bone and dermis show a decreased ability to dedifferentiate at late stages and that these mature tissues in turn induce "overproduction" of cartilage and fibrous tissue. Forsyth also found strong indications that the tissues of non-regenerating limbs have lost their capacity to dedifferentiate. The findings in the present study regarding the proliferation of cartilage and its failure to dedifferentiate in the non-regenerating limbs would seem to lend support to this interpretation.

In this connection the work of Grobstein (1947) is of interest. He found that androgen is responsible for the morphogenesis of the gonopodium of a poeciliid fish and is also responsible for the loss of regenerative capacity of this organ. He pointed out the similarity between this situation and that which obtains in anuran limb regeneration and concluded that the effect of androgen in the one instance and thyroxin in the other is primarily morphogenetic, but that these hormones act as limiting factors on the regenerative process. He pointed out that this does not preclude the influence of a local factor as the immediate cause of regenerative failure since the morphogenetic effect actually consists of local changes in organs, tissues, cells, and at subcell levels which could be directly responsible for changes in regenerative ability. One possibility at the sub-cell level is suggested by the work of Weinstein (1940) who has demonstrated an effect of anterior pituitary extract upon the permeability of connective tissue ground substance. Whether similar effects could be obtained with thyroid hormone is not known, but it would seem that a study of this matter and of the possible relation between permeability of ground substance and regeneration would be of value.

Summary

1. Continued administration of thiourea to tadpoles of *R. pipiens* which possess the capacity for limb regeneration results in retardation of differentiation.

2. Administration of thiourea to tadpoles which are in early stages of metamorphosis results in slight acceleration of a macroscopic differentiation if the treatment is begun 10 days prior to amputation and is discontinued after this time.

3. Treatment with thiourea for 10 days before amputation results in an increased rate of blastema formation and possibly in an increased rate of cartilage dedifferentiation in regenerating limbs.

4. Thyroid inhibition with thiourea results in a decreased mitotic activity in the blastema.

5. Tadpoles which have lost the capacity for limb regeneration, failed to regenerate limbs following treatment with thiourea for 10 days prior to amputation. However, there is evidence that such treatment stimulated an attempt to form a blastema.

6. It is concluded that the tissues have undergone some change during morphogenesis which adversely affects their blastema forming ability, but that treatment with thiourea favors an attempt to form a blastema.

References

1. Borssuk, R. A. 1936. Untersuchung des Verlustes der Regenerations-sfahigkeit der hinteren Extremitat von *Rana temporaria*. *Arch. Entw. Mech. Org.*, **133**, 349-356.
2. Forsyth, J. W. 1946. The histology of anuran limb regeneration. *J. Morph.*, **79**, 273-322.
3. Ghidoni, M. 1948. Effects of thyroid inhibitors upon tail regeneration in the tadpole. *Growth*, **12**, 181-202.
4. Gidge, N. M., & Rose, S. M. 1944. The rôle of larval skin in promoting regeneration in adult anura. *J. Exp. Zoöl.*, **97**, 71-89.
5. Goodwin, P. A. 1946. A comparison of regeneration rates and metamorphosis in Triturus and Amblystoma. *Growth*, **10**, 75-87.
6. Grobstein, C. 1947. The rôle of androgen in declining regenerative capacity during morphogenesis of the *Platypoecilus maculatus* gonopodium. *J. Exp. Zoöl.*, **106**, 313-344.
7. Guyenot, E. 1927. La perte du pouvoir regenerateur des Anoures, etudies par la methode des heterogreffes, la notion de territoires. *Rev. Suisse Zoöl.*, **34**, 1-53.
8. Hall, A. B., & Schotté, O. E. 1951. Effects of hypophysectomies upon the initiation of regenerative processes in the limb of *Triturus viridescens*. *J. Exp. Zoöl.*, **118**, 363-387.
9. Liosner, L. D. 1931. Uber den Mechanismus des Verlusts der Regenerationsfahigkeit wahrend der Entwicklung der Kaulquappen von *Rana temporaria*. *Arch. Entw. Mech. Org.*, **124**, 571-583.
10. Marcucci, E. 1916. Capacita regenerativa degli arti nella larve di Anuri e condizioni che ne determinano la perdita. *Archivio Zoöl.*, **8**, 89-117.
11. Naville, A. 1927. La perte du pouvoir regenerateur des Anoures etudiee par les homogreffes. *Rev. Suisse Zoöl.*, **34**, 269-284.
12. Pawlowsky, E. N. 1923. Hyperthyroidismus und Regeneration. *Arch. Entw. Mech. Org.*, **99**, 620-627.
13. Polezaiev, L. V. 1936. Sur la restoration de la capacite regenerative chez les Anoures. *Arch. Anat. Micr.*, **32**, 437-463.
14. ————. 1946. The loss and restoration of regenerative capacity in the limbs of tailless amphibia. *Biol. Rev.*, **21**, 141-147.

15. ———, & GINSBERG, G. I. 1939. Studies by the method of transplantation on the loss and restoration of the regenerative power in the tailless amphibian limbs. *C. R. Acad. Sci. URSS,* **23**, 733-737.

16. ROSE, S. M. 1948. Epidermal dedifferentiation during blastema formation in regenerating limbs of *Triturus viridescens*. *J. Exp. Zoöl.*, **108**, 337-361.

17. RUGH, R. 1951. *The Frog*. The Blakiston Co., Philadelphia; Toronto.

18. SCHOTTÉ, O. 1926. Hypophysectomie et regeneration chez les Batraciens urodeles. *C. R. Soc. Physiol. Hist. Nat. Geneve,* **43**, 67-72.

19. ———, & HARLAND, M. 1943. Amputation level and regeneration in limbs of late *Rana clamitans* tadpoles. *J. Morph.,* **73**, 329-363.

20. SEIGEL, S. 1951. The effect of thyroxin upon the rate of limb regeneration in the tadpole. Master's Dissertation, The Catholic University of America.

21. SPALLANZI, L. 1769. *An Essay on Animal Reproduction*. T. Becket and P. A. Hunt, London. 86 pages.

22. SPEIDEL, C. C. 1929. Studies of hyperthyroidism. VI. Regenerative phenomena in thyroid treated amphibian larvae. *Amer. J. Anat.,* **43**, 103-165.

23. WALTER, F. K. 1911. Schilddruse und Regeneration. *Arch. Entw. Mech. Org.,* **31**, 91-130.

24. WARREN, E. A., & BOWER, C. M. 1939. The influence of normal and induced metamorphosis on hind limb regeneration in *Rana sylvatica*. *Anat. Rec.,* **73**, 55-56.

25. WEINSTEIN, L. 1940. Further studies on the prophylaxis of experimental infections and intoxications with various hormone preparations. *Yale J. Biol. & Med.*, **12**, 549.

Growth, 1953, **17**, 45-65.

STUDIES ON TRANSPLANTED EMBRYONIC LIMBS OF THE CHICK. V. THE PATHOLOGY IN POST-HATCHING STAGES[1, 2]

HERBERT L. EASTLICK AND JOHN D. CHEHAK

Department of Zoölogy, The State College of Washington, Pullman, Washington

(Received for publication January 7, 1953)

INTRODUCTION

The various phases of development and differentiation of embryonic avian-limb grafts have been described by several workers, including Hunt (1932), Hamburger (1933, 1938), and Eastlick *et al.* (1943, 1944, 1947). Mammalian-tissue grafts have been studied extensively by Loeb (1945), who has shown that the host-graft reaction varies considerably, depending on the species relationship between host and donor tissue, and that the severity of the reaction can be judged by the type and number of invading leucocytes and also by the amount of connective tissue replacing the grafted tissue. Kozelka (1929), studying homoplastic integumental grafts in the domestic fowl, noted a host-graft incompatibility, the reaction being characterized by excessive vascularization and swelling. These grafts were either replaced by connective tissue or were completely absorbed. Macroscopic observations have been made by Eastlick (1941) on interspecies avian-limb grafts. Such grafts displayed an incompatibility reaction within a rather definite time after hatching, the time varying with each species studied. Indications of the reaction were edema, hemorrhage, scabbing, and vesiculation of the epidermis, and general necrosis followed by absorption or loss of the grafted limb. In some cases, however, healing took place only to be followed by another reaction which destroyed the transplant.

Since very little has been written on the pathology of transplanted avian-limb bud grafts following the hatching of the hosts, it is the

[1]This investigation was supported in part by the State College of Washington Committee on Research and by a grant from the Rockefeller Foundation.

[2]The authors acknowledge the helpful criticism of Drs. D. R. Cordy, C. H. Drake, D. S. Farner, and M. T. James during the course of the study.

objective of this paper to describe the various changes which occur in such a series of grafts.

Materials and Methods

White Silkie and New Hampshire chick embryos were used interchangeably as hosts and donors. The age of the embryos at the time of transplantation was usually 3-3½ days. The posterior limb buds were transplanted according to a modification of the method described by Hamburger (1933, 1938).

This report is based on 26 grafts which ranged from 4 days post-hatching to a maximum of 2 years of age. Insofar as possible, tissue was taken from six positions on each graft, namely the graft attachment, the lateral, medial, and ventral aspect of the first segment, and the lateral and medial aspects of the second segment. In the youngest grafts, and in severely incrusted or necrotic grafts, it was impossible to obtain tissues from all of the above mentioned positions, but representative sections were examined.

After the hosts were decapitated, the tissues were excised from the grafts, fixed in Formol-Zenker or Bouin's fixatives, dehydrated, cleared in xylol, embedded in Watterman's mixture or 54° to 56° C paraffin, and sectioned, usually at 8-10 micra. Sections were routinely stained in Harris' hematoxylin and eosin, although Pollak's trichrome stain, Delafield's hematoxylin, hematoxylin-eosin-azure II and Mallory's triple stain were used. The Congo red method of testing for amyloid was employed on some sections.

Observations

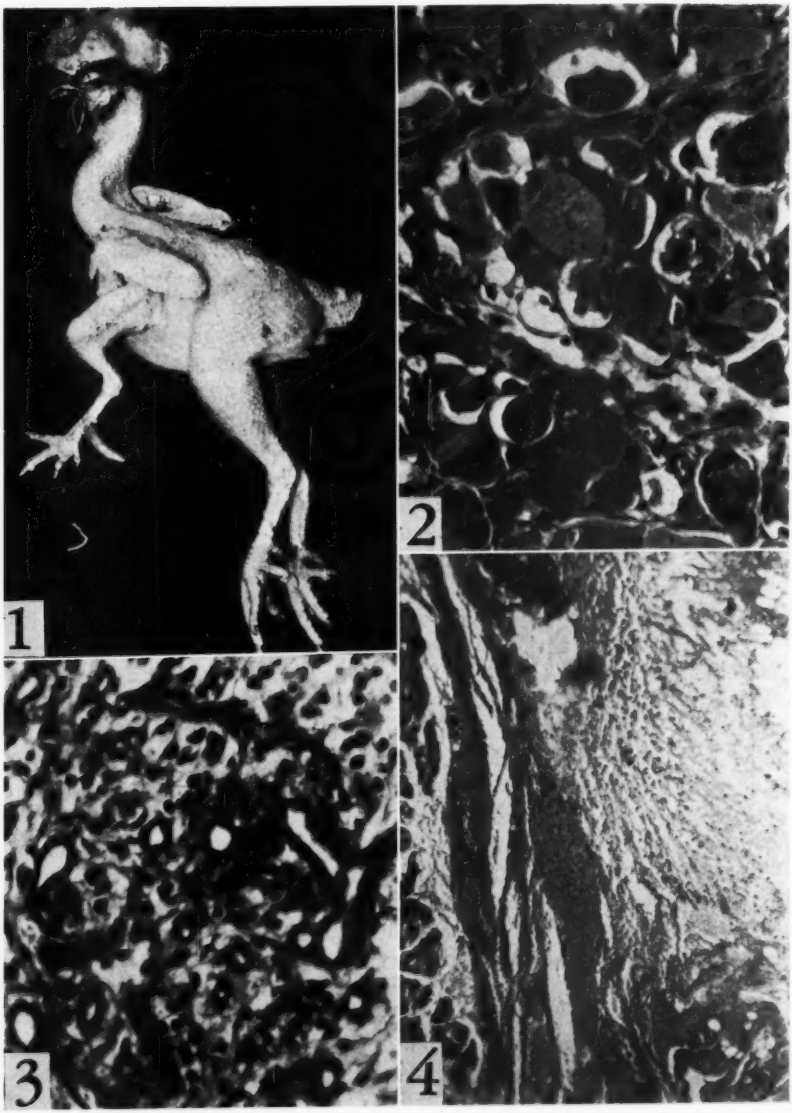
For the most part the grafts were normal in shape at the time of hatching, but two (172 and 207), were abnormally formed. The feathers were normal in number and distribution and the limb segments were reasonably proportional to those of controls but invariably reduced in size (Figure 1). One graft (100) displayed an increased amount of bone or cartilage leaving only a scant amount of connective tissue and muscle covered by normal epithelium.

The first indication of a host-graft incompatibility was a slight roughening and scaliness of various portions of the skin. Two grafts (147 and 152) became denuded and the skin appeared yellowish, and soon swelling and edema occurred. Occasionally a simultaneous thickening of the skin occurred in edematous areas. Eventually the

pressure of the edema fluid ruptured the skin and various types of exudates appeared. The exudates ranged from serous to sero-hemorrhagic, and where bacterial invasion of the ruptured skin occurred, a pasty, purulent exudate was formed. In some areas, pieces of skin sloughed and the feathers dropped out. When the remaining feathers of an incrusted area were pulled, the skin and scab frequently came off together. Necrosis proceeded proximally from the toes toward the place of attachment and frequently the foot shriveled and eventually dropped off. Some grafts were almost entirely denuded of skin and feathers and an incrusted exudate covered the appendage. In the majority of grafts, evidence of this reaction is first seen on the distal segments. Reduction of edema, decreased incrustation and development of new epithelium and feathers takes place in those grafts which survive the reaction.

Definite incompatibilities were seen macroscopically as early as 3 weeks after hatching and as late as 1½ years, but there is histological evidence that the reaction may begin as early as 4 days after hatching. The grafts were also found to display a rather severe reaction microscopically when there was little or no sign of a gross reaction. When the gross reaction was apparent, destruction of the graft was well underway. By examination of the 5 or 6 pieces taken from each individual graft, the progressive steps of the reaction can be more clearly followed. When a gross reaction was apparent, it was most severe distally in all but one case.

The destruction of striated muscle appears to be the first pathological change in the grafts. Although the form of muscle breakdown may differ between individual grafts, the majority of the muscle is replaced by other types of tissue. First evidence of injury to the striated muscle is a misalignment of the striations of adjacent myofibrils. Following this the muscle fibers show varied staining reactions (Figure 2). Another indication of injury at this period is the clumping of muscle nuclei within the fiber, particularly in areas adjacent to capillaries which may transect the fibers or in areas undergoing sarcolysis. Occasionally myofibrils swell but this does not appear to be the general rule. Eventually the striations begin to disappear and several different forms of destruction occur. Some muscle displays a gradual fading and dissolution (sarcolysis). Sarcoplasm is released in the affected area and is generally phagocytized by macrophages. Occasionally sarcolysis will progress to the point where only


1
2
3
4

EXPLANATION OF PLATE I

FIGURE 1

Graft Fls 124. Age: 4 years at autopsy. The left posterior limb bud of a 34 somite donor was transplanted to the left flank of a 38 somite host. Ordinarily the transplant is placed on the right flank for the chick embryo normally lies on its left side. This graft never gave external evidence of an incompatibility reaction. Note normal proportions but reduced size of the graft.

FIGURE 2

Graft 138. Age: 12 weeks. This section is from the lateral aspect of the first segment. The muscle fibers are disintegrating. Many of the fibers are poorly stained and some fibrillae are becoming clumped. Note that some fibrillae have shrunken away from the sarcolemma. The endothelial cells are swollen and the capillaries are enlarged and ramified. Harris' haematoxylin and eosin. X430.

FIGURE 3

Graft 152. Age: 9 weeks. This section is from the region of the host-graft attachment area. The muscle has disintegrated and the capillaries are dilated and ramified. Numerous macrophages and lymphocytes are present. Harris' haematoxylin and eosin. X430.

FIGURE 4

Graft 79. Age: 9 weeks. This section is from the region of the host-graft attachment. Muscle bundles are shown on the left followed by fibrous connective tissue, loose connective tissue which contains macrophages and lymphocytes, an edematous area and lastly adipose tissue. Harris' haematoxylin and eosin. X100.

the sarcolemma and bordering muscle nuclei are seen. The muscle, on losing its striations, may hyalinize. Hyalinized muscle, and also some that is not hyalinized, may show a peculiar vacuolation, beginning from the center of an individual muscle fiber. The vacuoles are evidently formed by a process of dissolution but differ from the preceding process in that only local areas of the fiber are affected and no fading of the staining reaction occurs around the vacuolated area. Some of the hyalinized muscle occasionally breaks into small fragments, dissolves out or is attacked by macrophages.

Some muscle fibers appear to fragment into rounded masses of sarcoplasm which generally take a basophilic stain. The masses give a highly stippled appearance and their size is quite variable. The staining reaction of the masses gradually fades, the muscle fibers disappear, and only a few fibroblasts and lymphocytes remain.

Frequently the sarcoplasm is seen to shrink away from the sarcolemma (Figure 2). Shrinkage may proceed to such an extent that the fiber appears to be less than half of its original size. This condition appears unrelated to atrophy, as the sarcolemma retains the original size and no relative increase in number of nuclei is observed. The process is probably more intimately associated with sarcolysis.

Muscle nuclei appeared resistant to the destructive process overtaking the fibers. Many areas in which the myofibrils had undergone sarcolysis contained normal nuclei, either free or still bordering the remaining sarcolemma. In the later stages of the reaction or when the breakdown of muscle was overwhelming, the nuclei displayed pyknosis or chromatolysis.

All of the above changes have been noted at varying ages and have occurred without a cellular invasion with the exception of a few macrophages in muscle undergoing sarcolysis. Furthermore, all parts of a muscle do not break down simultaneously. One fiber may be severely affected by sarcolysis or vacuolation, while adjacent fibers appear normal. In most cases it appears that the fibers are indiscriminately affected, and a position relationship was not observed.

At this stage of the reaction there is a very limited destructive process taking place in the smooth muscle of the feather. A few grafts had small (2-3 micra) refractile bodies surrounding the nuclei of the smooth muscle. The droplets gave a beaded appearance and their size was constant. The droplets are the first step in a destructive process of smooth muscle and will be mentioned in the description

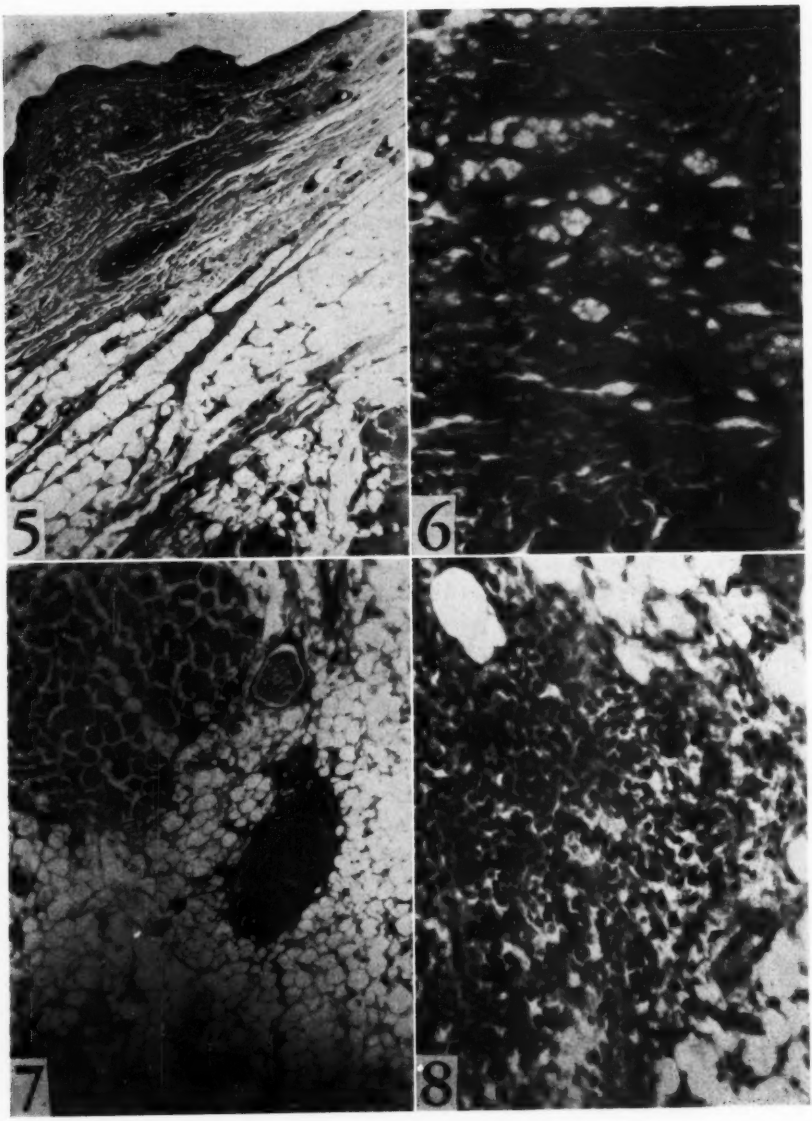
of a later stage of the reaction when smooth muscle destruction is more prevalent.

Along with early destruction of striated muscle there is an associated vascular reaction. The capillaries are moderately dilated and some of the larger vessels are distended (Figure 3). The hyperemic vessels were generally seen in muscle or in areas where muscle remnants were being phagocytized by macrophages. Occasionally the capillaries and arterioles of the subcutis were hyperemic. Some areas showed hemorrhage by diapedesis, but only rarely did a vessel rupture. Slight edema between muscle fibers and in subcutaneous areas was seen.

When muscle breakdown and capillary proliferation increases, it appears that the reaction may take at least two separate courses: either the destroyed muscle is replaced by adipose tissue or fibrosis occurs in the areas from which muscle is removed. Development of adipose tissue and fibrosis may occur simultaneously within the same section (Figures 4, 5).

Replacement of muscle by adipose tissue is the more common phenomenon, at least in the younger grafts. As the capillaries enter the area undergoing muscle destruction, embryonic lobules which are composed of multivacuolated cells are seen in the immediate vicinity of capillaries (Figure 6). It appears that these lobules develop from a syncytium of mesenchymal and reticular cells. In time a very considerable portion of the graft may be replaced by adipose tissue. As the fat lobules are formed, small accumulations of lymphocytes appear around the capillaries of the subcutis. At times the lymphocytes are diffusely distributed throughout the subcutis, but in general they remain closely associated with the capillaries.

Mature adipose tissue soon replaces much of the destroyed muscle (Figure 7). However, the formation of adipose tissue and its replacement of muscle may be restricted to only certain parts of the graft. It appears that the greater number of fat lobules occur in the more proximal regions of the grafts. The presence of mature adipose tissue appears to be associated with a definite increase of infiltrating cells, especially in subcutaneous tissue and adipose tissue. The capillaries of the adipose tissue are at first hyperemic. Shortly afterwards the congestion decreases and is followed by an infiltration of lymphocytes between the fat lobules (Figure 8). As the number of lymphocytes in the adipose and subcutaneous tissues increase, the reaction within


5
6
7
8

EXPLANATION OF PLATE II

FIGURE 5

Graft 502. Age: 15 weeks. This is a section through the medial aspect of the second segment. The muscle beneath the dermis is being replaced, predominantly by adipose tissue, but some fibrous connective tissue is present. Muscle bundles which are beginning to disintegrate occur at the lower right border. Note exudate above the epidermis. Harris' haematoxylin and eosin. X100.

FIGURE 6

Graft 129. Age: 3 weeks. This section is from the ventral aspect of the first segment. This is an early stage and the replacement of disintegrating muscle by multivacuolated fat cells. Numerous macrophages are present. Harris' haematoxylin and eosin. X430.

FIGURE 7

Graft 502. Age: 15 weeks. This is a section through the medial aspect of the second segment. Intact muscle, a normal appearing cross section of nerve, an aggregation of macrophages, numerous lymphocytes, and plasma cells surrounded by adipose tissue are readily noted. Harris' haematoxylin and eosin. X430.

FIGURE 8

Graft 207. Age: 6 weeks. This is a section of the lateral aspect of the first segment. Macrophages, lymphocytes and plasma cells surrounded by adipose tissue are noted Harris' haematoxylin and eosin. X430.

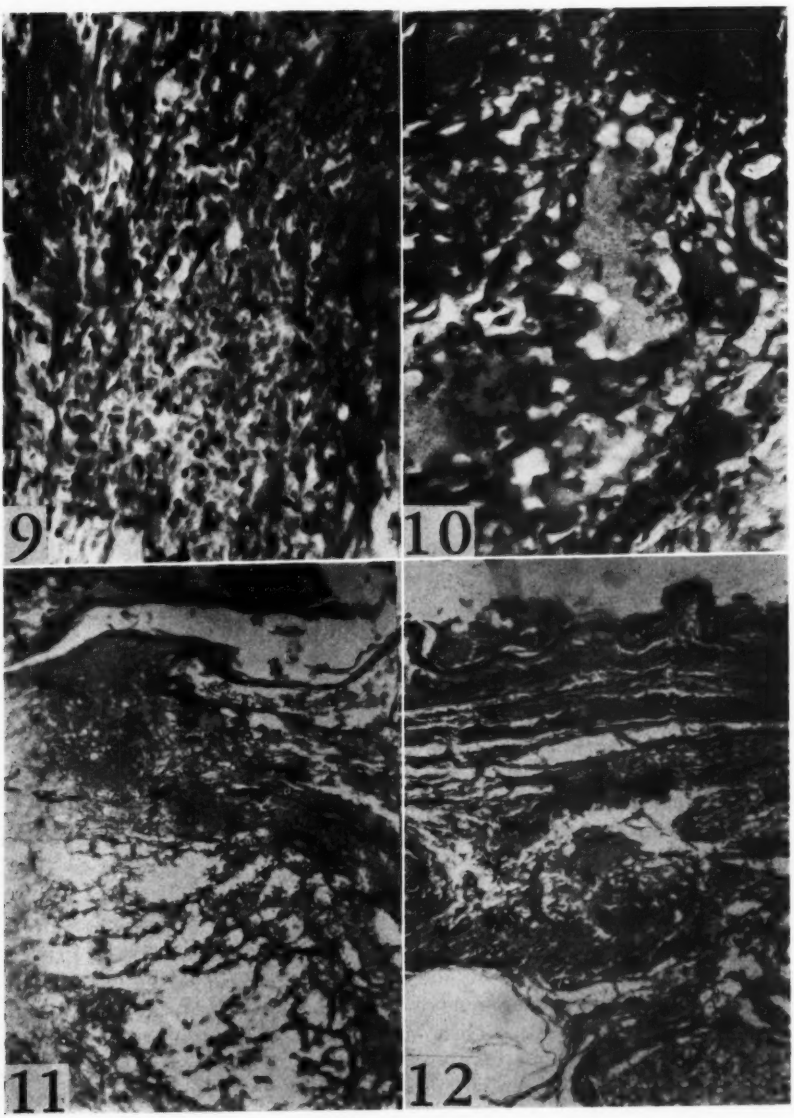
the remaining muscle is noticeably hastened. The vessels are more congested and hemorrhage occurs both by diapedesis and also by direct rupture of some of the larger vessels (Figures 9, 10). Lymphocytes also enter the muscle, but generally are not accompanied by erythrocytes. One part of the muscle may show severe hemorrhage while another part seems to have only a selective infiltration of lymphocytes. The lymphocytes appear to enter the area of muscle which has already started to break down. Damage to blood vessels is noted during this stage. The endothelium first becomes roughened and the endothelial cells imbibe fluid and swell. Eventually the cells of the tunica media and tunica adventitia also imbibe fluids and the entire wall of the vessel appears heavily vacuolated. The degree of damage to the blood vessels seems associated with the severity of edema in the surrounding tissue.

Fibrosis is evident in tissues which have undergone the reaction for a considerable period of time. Fibroblasts proliferate and lay down varying amounts of connective tissue in areas where muscle has been or is being destroyed by phagocytosis and sarcolysis. In graft 152 young fibroblasts were present in an area along with a flood of hemorrhaged blood (Figure 9). They presumably would have formed connective tissue as the blood was removed by macrophages. Generally, however, young connective tissue replaces striated muscle or the original connective tissue of the subcutis. Young connective tissue may persist for some time, but if conditions are favorable, more collagen fibers will be formed and eventually a large portion of the graft will be occupied by a densely packed, mature connective tissue. Fibrosis may proceed to such an extent that the larger blood vessels become completely fibrotic and the lumina of the vessels are obliterated. Rarely was there any evidence that adipose tissue was being replaced by connective tissue for, in most cases, adipose tissue replaced the connective tissue.

Although edema is consistently present throughout the earlier stages of the grafts, its degree has not been sufficient to affect the tissues seriously. In the later stages of the reaction its effect cannot be underestimated. Severe edema generally occurs where the loose fibrillar type connective tissue is forming (Figures 11, 12). The edema fluid first appears around the larger vessels which, on many occasions, appear badly damaged. It is also at this point that the cells of the blood vessels imbibe the greatest amount of fluid. As the edema

fluid floods an area, the young fibroblasts become pyknotic and connective tissue proliferation ceases. If adipose tissue is present, it too, is flooded by the fluid, especially between the lobules. Young fat lobules are not seen when severe edema is present, but at no time was there any evidence of fat necrosis in the form of fatty acid crystals or calcium soaps. Macrophages and plasma cells imbibe much fluid and then rupture, becoming part of the exudate. Lymphocytes appear to become pyknotic with the advent of the edema fluid. Hemorrhage in the surrounding areas is definitely increased and blood plus the edema fluid may form a sero-hemorrhagic exudate in the severe cases. Occasionally a fibrin network is formed. If the edema decreases, fibroblasts enter the area and fibrosis begins. Generally the edema appears to continue. It pushes toward the surface carrying with it necrotic cellular and tissue elements. These elements are banked against the basal layer of the epithelium and remain there until the pressure of the edema fluid is such that the epithelium is ruptured. It is evident that the substances contributing to the edema fluid are variable. Commonly the fluid is clear, taking very little stain. In other instances its staining reaction becomes increasingly eosinophilic. In grafts 152 and 149 the edema fluid flooded a muscle area and was tentatively identified as an amyloid deposit, but a test for amyloid proved negative. It appears more likely that an eosinophilic edema fluid is indicative of a high protein content. On occasion the edema fluid appears basophilic. This gives an appearance similar to that of mucoid degeneration.

As edema becomes increasingly noticeable in the deeper tissues, a marked increase in the thickness of epithelium is seen. Mitoses in the stratum germinativum are numerous and keratinization is increased. The cells of the germinativum may take on a fusiform shape and extend a considerable distance into the dermis, as if to serve as a foothold for the epithelium. When the edema fluid reaches the epithelium, it generally squeezes through the intercellular spaces of the germinativum and is imbibed by the more peripheral cells. Eventually the pressure of the edema fluid and the vesiculation of the epidermal cells is so great that the weaker spots of the epithelium rupture (Figure 11). Edema fluid and cellular debris are forced through this opening and an incrusted area forms on the surface. This process may slacken and new epithelium attempts to cover the defect. The new epithelium was never seen to attain a thickness of more than 2 cells.


9
10
11
12

EXPLANATION OF PLATE III

FIGURE 9

Graft 152. Age: 9 weeks. This is a section of the lateral aspect of the first segment. A hemorrhage has occurred in the central area and lymphocytes are infiltrating the area. Harris' haematoxylin and eosin. X430.

FIGURE 10

Graft 32. Age: 6 weeks. This is a section in the region of the host-graft attachment The muscle has disintegrated. A hemorrhage has occurred and numerous lymphocytes are invading the upper right position of the field. Harris' haematoxylin and eosin. X430

FIGURE 11

Graft 3253. Age: 15 weeks. This is a section of the medial aspect of the first segment. A thick exudate overlies the vesiculated epidermis. The subepidermal area is edematous. Harris' haematoxylin and eosin. X100.

FIGURE 12

Graft 207. Age: 6 weeks. This is a section from the region of the host-graft attachment area. Note superficial exudate, exfoliating epidermis, and hemorrhagic, edematous subdermal area. Harris' haematoxylin and eosin. X100.

On several occasions the reaction appears to have stimulating effect on the epithelium. Long finger-like projections of epithelium extend into the dermis and may cover a considerable area. Some of the projections are isolated as islands and eventually form epithelial pearls.

The defect caused by the ruptured epithelium may not close over. In this case the graft is subjected to bacterial invasion, a condition which may play an important rôle towards final destruction of the graft. Even though the graft is severely affected by the incompatibility reaction, it is still healthy enough to present the normal picture of an acute inflammation due to bacteria. Lymphocytes and plasma cells are replaced by a mass infiltration of neutrophils or younger myeloid elements. A fibrin network is formed and young fibroblasts eventually enter to attempt healing of the abscess.

It was previously mentioned that smooth muscle was, on occasion, mildly affected in the early part of the reaction. This was characterized by an accumulation of small refractile droplets around the nucleus. It is difficult to determine whether these droplets are a product of muscle-cell fluids or whether the production of the surrounding edema fluid is an influencing factor in muscle deterioration. The muscle is not further affected until the surrounding reaction becomes severe. Frequently the smooth muscle remains almost completely unaffected even though adipose tissue has replaced the entire section or edema and hemorrhage are prevalent. When smooth muscle does break down, it appears as though the droplets coalesce to form vacuoles which may or may not be filled with fluid. Eventually, however, irregular spaces are left in the tissue, a condition similar to that in the striated muscle. The muscle nuclei become pyknotic and the cytoplasm shows variable staining reactions. In a few instances, intact smooth muscle is invaded by lymphocytes. In time the smooth-muscle bundles are entirely destroyed and the area is replaced by adipose tissue or connective tissue. It appears that smooth muscles of feathers are one of the most resistant tissues in the grafts.

Nerve trunks also seemed to be resistant to the reaction. They appeared normal in grafts which were severely fibrotic or edematous. At no time were the nerves ever invaded by leucocytes, although occasionally accumulations of mononuclear cells were seen to surround them. Infrequently a nerve in cross section was rarefied and ragged, but the significance of this was not determined.

The histological as well as the gross findings were for the most part in accord with one another, so far as the position of the severest reaction is concerned. The reaction was always more intense in the distal portions of the graft; sections of the proximal portion of the graft always contained more muscle and gave evidence of less edema and less infiltration of cellular elements.

Discussion

The results of this study indicate that a progressive series of events takes place in the destruction of chick-limb grafts. Muscle is first destroyed by sarcolysis or vacuolation. Hyperemia and slight edema are closely associated phenomena, but it cannot definitely be determined whether they precede initial muscle destruction, occur simultaneously with it, or are a final result. Macrophages enter the area and are followed by varying degrees of capillary proliferation. The destroyed muscle is commonly replaced by adipose tissue, but in some cases fibrosis occurs (Figure 4). Lymphocytes enter the adipose tissue, subcutaneous tissues and areas of fibrosis; plasma cells are also widely distributed (Figure 8). These cells are commonly found throughout areas of young and old connective tissue. Larger blood vessels become fibrotic or the cells of the vessel wall imbibe fluid and the vessel degenerates. The accumulation of edema and cellular debris eventually causes swelling of the epithelial cells. The epithelium then ruptures, laying open the way for additional destruction by bacteria.

In chick-limb grafts, where muscle, bone, connective tissue and other elements are present, there is probably a greater complexity to the reaction than that which occurs in grafts of only a single tissue. The histological descriptions of grafted tissues presented by other authors, although varying in details, are similar in their basic content. Murphy (1926) found that the reaction to foreign tissues was characterized by edema, mononuclear infiltration and fibrosis. These findings are in accord with those of Loeb (1945) who, in addition, found that different tissues such as cartilage, thyroid and spleen produced different degrees of intensity of the reaction. Sandstrom and Kauer (1933), who transplanted macerated duck kidney tissue to the chorio-allantoic membrane of the chick, suggested that the antagonistic reaction manifests itself by the invasion of granulocytes and connective tissue. Elson (1929) studying homoio-transplants of

striated muscle in the rat, observed a rather intense lymphocytic reaction as early as six days after transplantation and, in time, complete obliteration of the muscle by lymphocytes. A report of tissue reactions in the transplantation of muscle in urodeles has been made by Horowitz (1937) who notes that fibroblasts first invade the transplant, later to be followed by lymphocytes which were said to destroy the graft. Collagen has been transplanted under the skin by Pullinger and Pirie (1942) and a detailed histological study has been made of the ensuing inflammatory process. A vascular connective-tissue capsule formed about the graft and subsequent invasion of neutrophils and mononuclear cells took place. Strangely enough these cells became pyknotic and disappeared from the graft. The collagen, however, was not removed and remained healthy until the time macrophages, plasma cells and lymphocytes again invaded the graft. Eventually foamy macrophages and lymphocyte nodules were seen and the graft was destroyed.

It is apparent from these studies that great stress is put on the fact that cells of the mononuclear series have an actual destructive effect on the graft. In the present study, however, this does not seem to hold true. Muscle was seen to be destroyed without an invasion by mononuclear cells, a condition strongly suggesting other factors. Further evidence of the lack of destructive power of the lymphocytes is seen in the fact that although adipose tissue was heavily infiltrated, at no time was there evidence of fat necrosis. In fact, areas of adipose tissue heavily infiltrated with lymphocytes showed an active development of young fat lobules. Eakin and Harris (1945), who studied xenoplastic grafts in amphibians, noted a certain correlation between the degenerative process and lymphocytes. These investigators are hesitant to attribute a destructive function to the lymphocytes and suggest that these cells may be only a secondary factor.

The entire situation is suggestive of at least two other possibilities concerning the function of lymphocytes in the graft. The mononuclear cells could serve in a protective or perhaps in a stimulating capacity to the new tissues which are replacing the old ones. The fact that new tissue is constantly being formed in the presence of large accumulations of these cells would seem to lend some support to this view. It is interesting to note that Carrel (1922) has placed extracts of leucocytes in tissue culture and found that a greater growth of the culture was promoted. Carrel is of the opinion that leucocytes secrete a growth promoting factor at the time of their death or when

they invade the tissue. Speidel (1938) has observed an infiltration of leucocytes within the sarcolemma of an injured muscle fiber. He believes that leucocyte activity in an injured muscle area is an aid to regeneration of the muscle.

For many years the seat of antibody production has been attributed to cells of the mononuclear series. Recently Bjorneboe, Gormsen and Lundquist (1947) have produced a plasma-cell reaction in adipose tissue of the renal sinus by intensive immunization with pneumococcus. The extracts of the plasma cell infiltrated tissue showed that a rich amount of antibody was present. Less than 10% of the cells present in the tissue were lymphocytes, the rest being plasma cells. These investigators concluded that plasma cells were the main source of antibodies. Furthermore, they believe that the lymphocyte has the potential of antibody production but antibody is not released until the lymphocytes assume the form of the plasma cell. Ehrich (1947) also has noticed a large production of antibody where a lymphocyte-plasma cell reaction is produced by a small dose of antigen. He also believes that finding lymphocytes and plasma cells in the intimal and adventitial regions of the blood vessels is a sign of antibody production. A similar condition has been reported by Hawn and Janeway (1947) in which bovine serum albumen was used as an antigen. In addition to a heavy infiltration of mononuclears into the blood vessels, these authors noted swelling of endothelial linings and collagen formation within arterial walls.

It is perhaps not unreasonable to view the mononuclear infiltration of tissues in the present study as an indication of antibody production. The graft itself can be considered a foreign protein. The degenerating muscle or the edema fluid may have sufficient antigenic properties to call forth eventually an antibody response from the circulating mononuclear cells, especially the plasma cells which are so widely dispersed throughout the tissues. If this were true, no demonstrable antibodies would be seen in the early part of the reaction, rather in its later stages when plasma cells are more abundant. Kozelka (1933) has pointed out that in milder forms of tissue antagonism the host is able to eliminate the incompatible elements without destroying the tissue. This in turn could explain the function of the mononuclear cells, namely, to eliminate the incompatible elements. Eastlick (1941) has suggested that toxic substances may not be released to any extent from intact cells, and thus the reaction is delayed until

initial breakdown of the tissue. If the incompatible elements are set free upon breakdown, an antigen-antibody reaction might take place as the neutralizing process. If the entire reaction failed, a certain toxicity could be set up by the incompatible elements in which case severe edema and hemorrhage would occur with eventual destruction of the graft.

If the mononuclear cells invade the grafts and furnish antibodies for the neutralization of the toxic products resulting from tissue destruction, the host in turn would be protected. This could explain why the hosts were so little affected by the reaction in the limb graft.

An equally intriguing problem is presented when the initial breakdown of striated muscle is considered. The histological examination of the limb grafts in this study revealed all of the areas where adequately vascularized but innervation varied. Although a few thrombi were present in a few of the younger grafts, the lack of blood does not seem to be an important factor in the early breakdown of the tissue. There is also the question why smooth muscle and nerve tissue are so highly resistant when the surrounding tissues are markedly affected.

Loeb and Wright (1927) have suggested that the severity of the reaction of the host against the transplant is correlated with the number of strange genes within the transplant. Species relationship presumably has an effect on the intensity of the reaction in transplanted tissues. Harris (1948) implanted chick heart subcutaneously in rats and found that the resulting rat serum had a damaging effect on normal chick heart cultures. He believes that this can explain the mode of action of cytotoxins against alien transplants. Cultures in which fibroblasts were present survived the rat serum better than those without. This has been interpreted as an indication of fibroblasts having a neutralizing effect on cytotoxins. In a previous paper Harris (1943) suggested that cytotoxins are typical antibodies and are not evoked until the transplant is composed of a sufficiently foreign protein to act as an antigen.

Hewitt (1934) has suggested that the failure of heterografts in amphibians is due to the inability of grafts to use the food supplied by the host. It appears that chick-limb grafts show very few signs of nutritional atrophy. The fact that muscle so readily breaks down suggests that the complexities of protein replacement and maintenance may be involved.

Why the tissues near the attachment of the graft to the host are less affected than those at the distal points is an interesting question. If the inciting agent is of hematogenous origin, why are the proximal segments less affected than the distal? Perhaps the tissues near the host more easily compensate for individual differences between host and donor or else there may be an actual mixture of the two tissues. If the latter were true, it is logical to assume that the reaction would be less severe. Compensation for host differentials is indicated by the fact that some grafts possess large amounts of adipose tissue at the proximal segments but still have reasonable quantities of striated muscle. From this it appears that muscle at one time was actively breaking down and was being replaced by adipose tissue. Eventually this situation stopped and the remaining striated muscle was unaffected.

Summary

The post-hatching stages of 26 chick-limb grafts have been studied. The age of the grafts which were sectioned varied from 4 days to 2 years. Gross signs of the host-graft incompatibility reaction were observed as early as three weeks and as late as 1½ years after hatching. The reaction was usually observed between 6 and 9 weeks post-hatching. A histological sequence of events contributing to the breakdown of chick-limb grafts is presented. It appears that muscle is first affected. Muscle which has undergone sarcolysis is removed by macrophages and eventually is replaced by adipose or connective tissue. Slight edema and hemorrhage occur and mononuclear cells make their appearance in the connective and adipose tissue. A slight infiltration of lymphocytes is found in muscle but only after the muscle has begun to break down. Lymphocytes form a nodular accumulation around the capillaries, and many plasma cells are diffusely spread throughout the tissues. In the later stages of the reaction, edema and hemorrhage occur. This leads to eventual rupture of the epithelium. Bacterial invasion then takes place through ruptured epithelium and may play a rôle in the destruction of the graft. Some grafts show signs of recovery such as reduction of edema, decreased incrustation and the development of a new epithelium with a full complement of feathers.

REFERENCES

1. BJØRNEBOE, M., GORMSEN, H., & LUNDQUIST, F. 1947. Further experimental studies on the rôle of the plasma cells as antibody producers. *J. of Immun.*, **55**, 121-129.
2. CARREL, A. 1922. Growth promoting function of leucocytes. *J. Exp. Med.*, **36**, 385-391.
3. EAKIN, R. M., & HARRIS, M. 1945. Incompatibility between amphibian hosts and xenoplastic grafts as related to host age. *J. Exp. Zoöl.*, **98**, 35-64.
4. EASTLICK, H. L. 1941. Manifestations of incompatibility in limb grafts made between bird embryos of different species. *Phys. Zoöl.*, **14**, 136-143.
5. ————. 1943. Studies on transplanted embryonic limbs of the chick. I. The development of muscle in nerveless and in innervated grafts. *J. Exp. Zoöl.*, **93**, 27-49.
6. ————, & ANDERSON, R. H. 1944. Studies on transplanted embryonic limbs of the chick. II. The development of limb primordia within the anterior chamber of the eye. *J. Morph.*, **75**, 1-9.
7. ————, & WORTHAM, R. A. 1947. Studies on transplanted embryonic limbs of the chick. III. The replacement of muscle by "adipose tissue." *J. Morph.*, **80**, 369-389.
8. EHRICH, W. E. 1947. The significance of the tissue reaction caused by antigens. *J. A. M. A.*, **135**, 94-97.
9. ELSON, J. 1929. Auto- and homoiotransplantation of cross striated muscle tissue in the rat. *Am. J. Path.*, **5**, 425-438.
10. HAMBURGER, V. 1933. A method of transplanting limb buds in chick embryos. *Anat. Rec.*, **55**, (suppl. p. 58).
11. ————. 1938. Morphogenetic and axial self-differentiation of transplanted limb primordia of 2-day chick embryos. *J. Exp. Zoöl.*, **77**, 379-399.
12. HARRIS, M. 1943. The rôle of humoral antagonism in heteroplastic transplantation in mammals. *J. Exp. Zoöl.*, **93**, 131-145.
13. ————. 1948. Specificity and mode of action of cytotoxins produced against alien transplants in rats. *J. Exp. Zoöl.*, **107**, 439-453.
14. HAWN, C. VAN Z., & JANEWAY, C. A. 1947. Histological and serological sequences in experimental hypersensitivity. *J. Exp. Med.*, **85**, 571-590.
15. HEWITT, D. C. 1934. Xenoplastic transplantation of amphibian eye rudiments. *J. Exp. Zoöl.*, **69**, 235-267.
16. HOROWITZ, N. H. 1937. Tissue reactions to the transplantation of muscle in the urodele, *Triturus viridescens viridescens*. *J. Exp. Zoöl.*, **76**, 105-115.
17. HUNT, E. A. 1932. The differentiation of chick limb buds in chorio-allantoic grafts, with special reference to the muscles. *J. Exp. Zoöl.*, **62**, 57-91.
18. KOZELKA, A. W. 1929. Integumental grafting in the domestic fowl. *J. of Heredity*, **20**, 2-14.
19. ————. 1933. Serological studies of tissue antagonism in the domestic fowl. *Phys. Zoöl.*, **6**, 159-184.
20. LOEB, L., & WRIGHT, S. 1927. Transplantation and individuality differentials in inbred families of guinea pigs. *Am. J. Path.*, **3**, 251-283.
21. ————. 1945. The Biological Basis of Individuality. Chas. C. Thomas Co. 711 pp.

22. MURPHY, J. B. 1926. The lymphocyte in resistance to tissue grafting, malignant disease, and tuberculous infection. Monograph 21, Rockefeller Instit.

23. PULLINGER, B. D., & PIRIE, A. 1942. Chronic inflammation due to implanted collagen. *J. Path. and Bact.*, **54**, 341-344.

24. SANDSTROM, C. J., & KAUER, J. T. 1933. The growth and differentiation of macerated embryonic duck kidney tissue on the chorio-allantoic membrane of the chick. *Anat. Rec.*, **57**, 105-117.

25. SPEIDEL, C. C. 1938. Studies of living muscles. I. Growth, injury and repair of striated muscle, by prolonged observations of individual fibers in living frog tadpoles. *Am. J. Anat.*, **62**, 179-235.

Growth, 1953, **17**, 67-73.

STUDIES ON THE GROWTH RATE OF A RHABDOMYOSARCOMA[1]

ELIZABETH ESHELMAN MILLER

Institute for Cancer Research and Lankenau Hospital, Research Institute, Philadelphia, Pa.

(Received for publication January 9, 1953)

INTRODUCTION

During the course of chemical fractionation studies on a mouse rhabdomyosarcoma (1), a method was needed for determining quantitatively the effect which these fractions when reinjected into mice might have on the tumor. Various procedures have been proposed for measuring the growth rates or comparative sizes of tumors. About 1935 Schrek (2, 3, 4, 5) described extensive quantitative and statistical investigations of the growth characteristics of the Walker rat tumor, the Flexner-Jobling rat carcinoma and the R39 rat sarcoma. His method is based on the finding by Mayneord in 1932 that the greatest diameter of the Jensen rat sarcoma is a linear function of the time after transplantation. Schrek plotted the cube root of the product of diameters taken in three dimensions against time. The growth curves of the Walker rat tumor and the Flexner-Jobling rat carcinoma were found to be linear, while the R39 rat sarcoma gave variable types of curves, which were classified as linear, curvilinear, regressive and irregular. The types of curves appeared to be associated with different degrees of immunity produced by the host animal to the tumor, the linear type representing the least immune response. By analysis of these curves, Schrek divides tumor growth into three components: (1) the latent period, which is the time between inoculation of the tumor and the appearance of a palpable lesion and is indicated on the graph as the point where the curve intersects the ordinate; (2) the growth impulse or growth constant, which is represented by the slope of the curve and may be calculated by a formula fitted to the

[1]This work was supported by the Women's Auxiliary of the Lankenau Hospital; research grants from the National Cancer Institute of the National Institutes of Health, Public Health Service and the American Cancer Society.

curve; (3) the immune response, which is evident only in the curvilinear types of curves. These methods have been applied to a mouse tumor used in our laboratory, and the results of these studies will be described here.

Materials and Methods

The tumor used for these experiments was one which arose from the injection of 20-methylcholanthrene into inbred C3H mice, and its method of induction and histological characteristics have been previously described (1). This tumor (MC1A) was carried through the first 65 serial transplant generations by Dr. E. U. Green. It has now been through 125 transplants, and both its external characteristics and histological structure have not changed appreciably during this time. Only C3H mice of the original line were used in these experiments.

Tumor implantations were made with a 13-gauge trocar, two tissue fragments being placed subcutaneously into the left side of the mouse. In order to obtain tumors which are measurable with accuracy, it is necessary to insure that they grow tightly against the skin and are not attached firmly to the body wall. To achieve this, care must be taken not to scrape or penetrate the body wall with the trocar.

The length, breadth and depth of the tumors were measured with calipers, as described by Schrek (2), at intervals of 3 days to 1 week. The first values were obtained about the 8th day after transplantation, and on the 26th to 28th day the animals were sacrificed for the use of the tumors in extraction experiments; therefore, only this period of growth was studied. Most of the time one mouse produced two or more tumors from an inoculation, and frequently a number of small tumors arose along the needle tract, in spite of the fact that a separate trocar was used for each inoculation. It was considered that the largest tumor was representative of the growth rate, and only this was measured. When in the early stages of tumor growth it was difficult to determine which was the largest tumor, measurements on several were carried along simultaneously, but only the data for the one which proved to be the largest were used in the calculation. For each set of measurements tumors in 9-14 mice were used. Hair was shaved from the tumor area to facilitate measurement.

Results

Figure 1 shows the results of the measurements of tumors of 9 individual mice which were inoculated the same day and measured at the same time. For visual convenience, the data for the individual mice are plotted in a staggered order, the abscissa being shifted 1 cm. for each curve. Open and closed circles are used on alternate curves so that points from one curve will not be confused with the others. It is apparent that the data for each mouse may be fitted to a straight line, and that the slopes of the lines vary relatively little. The distribution for males and females is random. Although the slopes, which represent the growth rate, vary only slightly, the intercepts show significant differences.

This is more readily seen in Figure 2 where the measurements of individual mice are plotted as dotted lines on a single ordinate. However, when the average of determinations on all nine mice was calculated and plotted, a straight line was obtained which intercepted the zero point. This average is shown by the solid line. The point at which the lines intercept the ordinate would indicate the latent period, and the fact that a negative latent period was obtained in some instances suggests that in some cases the tumor fragment inoculated did not degenerate, but remained and functioned as an intact tumor. The other possibility is, of course, that these lines should not be straight but should curve from the zero point. According to Schrek's findings the size of the inoculum affects the length of the latent period and not the slope of the line, that is, the growth rate.

Statistically it seems significant and suggestive of random variations in response, that in the experiment shown, a straight line intercepting the zero point was obtained when the average of the measurements of 9 mice were plotted, and that in 12 additional experiments covering groups of 9-14 mice straight lines were obtained whose intercepts fell within ± 2 days of the zero point.

It was found that tumors of groups of mice inoculated at different times showed differences in growth rates, as indicated by differences in the slopes of the average lines. This difference in tumor growth has been observed by many investigators, and numerous explanations have been offered for it (5). There is some evidence that age, sex, season of the year and state of nutrition of the animals affect the growth rate. Variations in the behavior of tumor cells, that is, cyclic changes in proliferative energy or cyclic changes in infectivity, have been held responsible by some workers.

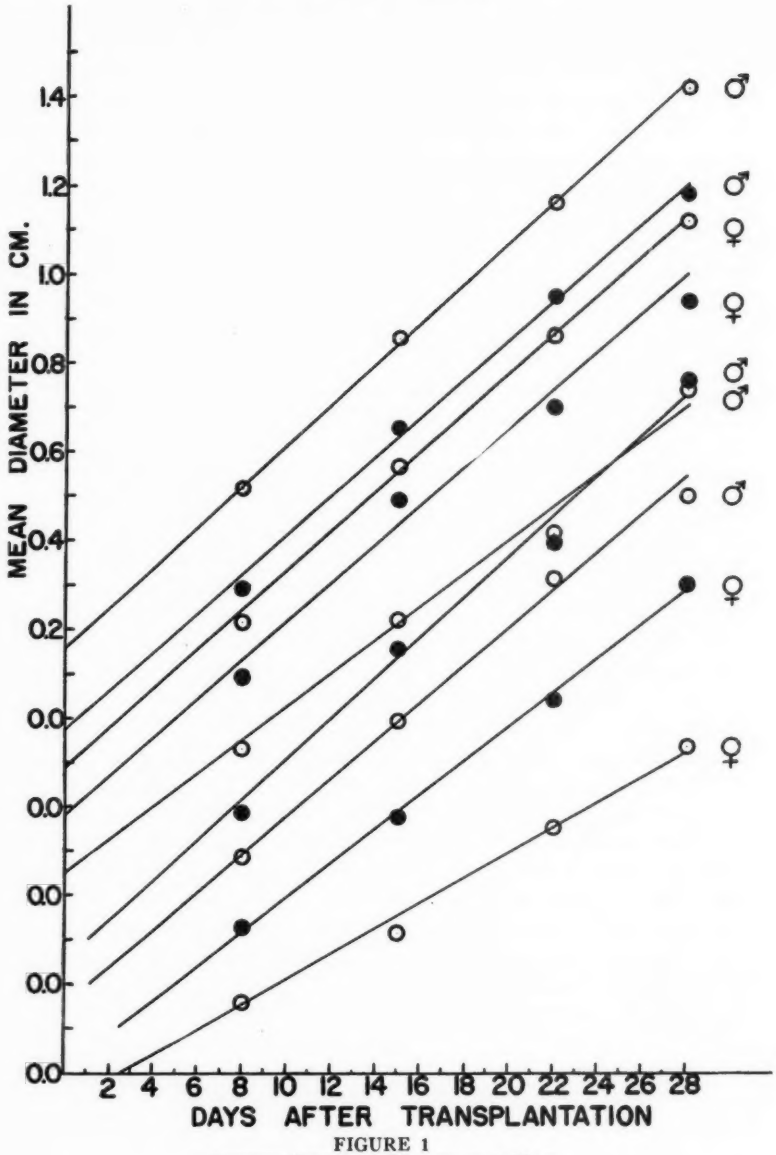


FIGURE 1
GROWTH CURVES OF TUMORS OF 9 MICE

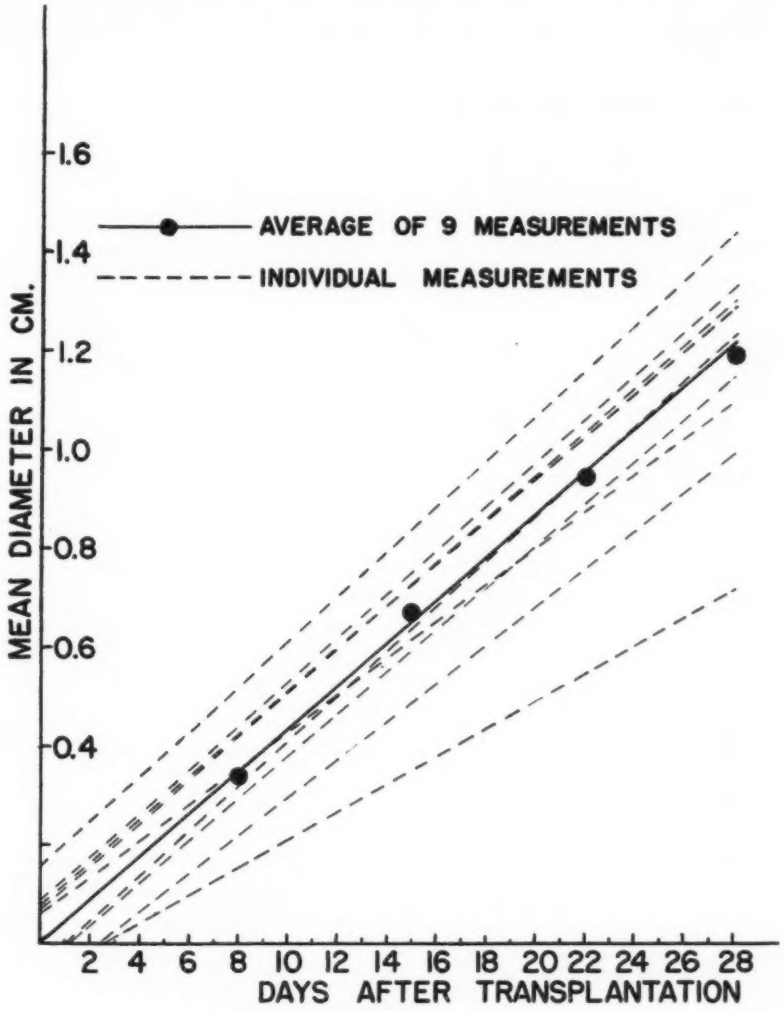


FIGURE 2

GROWTH CURVE CALCULATED FROM THE AVERAGE OF THE MEASUREMENTS OF THE TUMORS OF THE 9 MICE SHOWN IN FIGURE 1.

The slope of the individual curves is indicated by dotted lines.

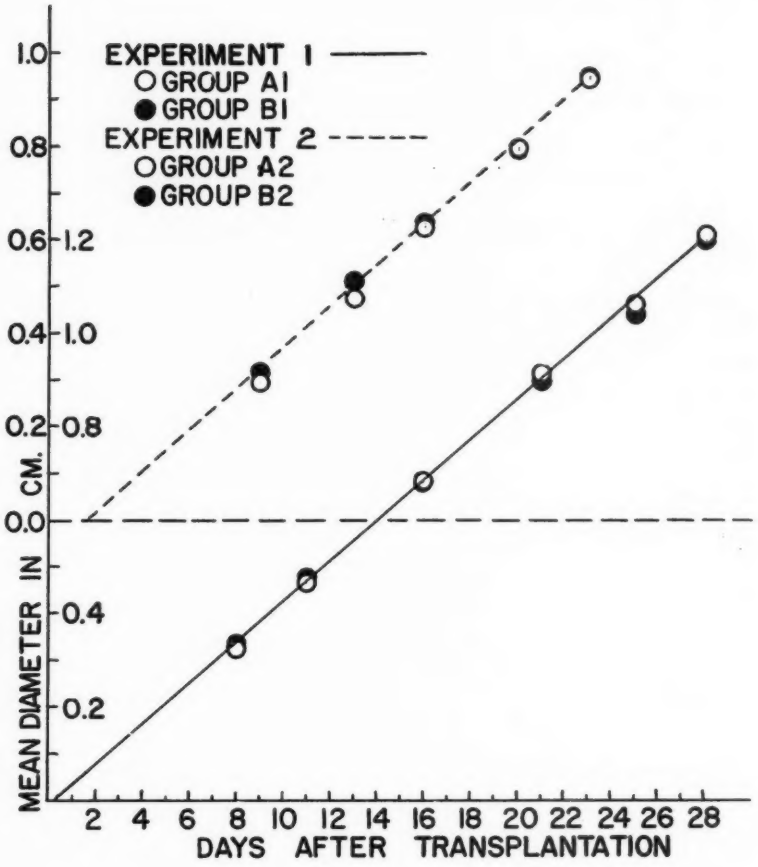


FIGURE 3

GROWTH CURVES OF TUMORS OF TWO GROUPS OF 10 MICE INOCULATED AND MEASURED AT THE SAME TIME

Although there is a significant difference in the growth of tumors of groups of normal mice inoculated at different times, this difference was minimized when two groups of 10 mice were inoculated simultaneously. This is indicated in Figure 3 where the average measurements for two experiments including two groups of 10 mice each are plotted on separate ordinates. In each experiment the two groups were divided on the basis of litters and sex, the same number of males and females from each litter being in each group. By this means the factors of age and sex are eliminated, and any variations in the growth rate of tumors due to slight genetic differences between individuals would average out.

In testing the effects of various substances on the growth of the tumors, the use of such paired groups for test and controls could give a valid comparison in experiments that were carried out at different times. A significant difference in the growth rate of the tumors in each pair of groups, as calculated from the slope of the line, would give an absolute basis of comparison for each phase of the test.

Conclusions

The growth of the transplantable MC1A rhabdomyosarcoma as measured by the rate of change of the cube root of the product of three dimensions of the tumor has been found to follow a straight line for at least 1-28 days after inoculation. The method used for measuring the growth rate has given reproducible results and appears to be suitable for studying the effect of various substances on tumor growth.

References

1. Miller, G. L., Green, E. U., Kolb, J. J., & Miller, E. E. 1950. Studies on the Proteins of Rhabdomyosarcoma and Normal Muscle of Mice. I. Gross Composition, Extractions with 0.5 M KCl, and Fractionations by Differential Centrifugation and Dialysis. *Cancer Research,* **10**, 141-147.
2. Schrek, R. 1935. A Quantitative Study of the Walker Rat Tumor and the Flexner-Jobling Rat Carcinoma. *Am. J. Cancer,* **24**, 807-822.
3. ———. 1936. Further Quantitative Methods for the Study of Transplantable Tumors. The Growth of R39 Sarcoma and Brown-Pearce Carcinoma. *Am. J. Cancer,* **28**, 345-363.
4. ———. 1936. The Effect of the Size of the Inoculum on the Growth Rate of Transplantable Rat Tumors. *Am. J. Cancer,* **28**, 364-371.
5. ———. 1936. Permanent and transient (fortuitous) variations of the growth components of transplantable rat tumors. *Am J. Cancer,* **28**, 372-388.

Growth, 1953, **17**, 75.

BOOK REVIEW

Growth of Children, by Alexander Low. University of Aberdeen, Scotland, 64 pages, 1952.

REVIEWED BY WILLARD C. OLSON

The importance of longitudinal records of growth of individual children has become apparent in research in child development. Such records are essential for studying problems of the individuality of patterns, the normality of deviations, and the possibilities of individual prediction.

The late Professor Low recognized this need and compiled measurements for sixty-six male and sixty female children at ages of three days and one, two, three, four, and five years. The tables have been prepared by Miss A. M. Clark, M. H. Quenouille, and R. D. Lockhart of the Anatomy Department of the University of Aberdeen.

The children were selected to be representative of North East of Scotland stock. Exact descriptions are given of the way each measurement was made. The cases are identified individually with the year of birth, the number of the pregnancy, the age of the mother, the age of the father, the month of cutting the first tooth, and information of whether bottle or breast fed. In addition to the gross measures of weight and height, one finds in these tables many detailed measurements of the head, the face, the trunk, and the extremities. Included also are counts of the eruption of the primary teeth. The mean values, standard deviations, and standard errors of the means are provided for each measure by sex and age.

The tables will be of interest in a practical way to pediatricians and dentists. Students of growth will have an opportunity to analyze the basic data provided in the tables so as to obtain deeper insight into the problems of individuality, continuity, and relationships in the growth process. Seriatim measurements on the growth of identical and fraternal twins and triplets from infancy to sixteen and twenty years are in preparation. A promise of a rich source for studies of family resemblance!